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<p>(21) International Application Number: PCT/US97/00760 (22) International Filing Date: 17 January 1997 (17.01.97) (30) Priority Data: 08/589,028 19 January 1996 (19.01.96) US (60) Parent Application or Grant (63) Related by Continuation US 08/589,028 (CIP) Filed on 19 January 1996 (19.01.96) (71) Applicants (for all designated States except US): BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US). BETAGENE, INC. [US/US]; Suite 125, 2600 Stemmons Freeway, Dallas, TX 75207 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): NEWGARD, Christopher, B. [US/US]; 4415 Ridge Road, Dallas, TX 75229 (US). HALBAN, Philippe, A. [CH/CH]; 41, chemin de Grange- Canal, CH-1208 Geneva (CH). NORMINGTON, Karl, D.</p>	<p>[US/US]; 10918 Aladdin Drive, Dallas, TX 75229 (US). CLARK, Samuel, A. [US/US]; 1603 S. Alamo, Rockwell, TX 75087 (US). THIGPEN, Anice, E. [US/US]; 9014 San Leandro, Dallas, TX 75218 (US). QUADE, Christian [DK/US]; 6258 Velasco Avenue, Dallas, TX 75214 (US). KRUSE, Fred [US/US]; 10438 Silverrock Drive, Dallas, TX 75218 (US). (74) Agent: HIGHLANDER, Steven, L.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: RECOMBINANT EXPRESSION OF PROTEINS FROM SECRETORY CELL LINES</p>		
<p>The diagram illustrates the genetic structure of the INS-1 locus. The top part shows the wild-type INS-1 allele with exons 1, 2, 3, and 4, and a probe region between exons 1 and 2. The middle part shows the INS-1 replacement vector with exons 1, 2, 3, and 4, and a probe region between exons 1 and 2. The bottom part shows the disrupted INS-1 allele with exons 1, 2, 3, and 4, and a probe region between exons 1 and 2. A 1 kb scale bar is provided.</p>		
<p>(57) Abstract</p> <p>The present invention provides methods for production of heterologous polypeptides using a variety of recombinantly engineered secretory cell lines. The common feature of these cell lines is the absence of expression of at least one endogenous polypeptide. The host cell machinery normally used to produce the endogenous polypeptide is then usurped for the purpose of making the heterologous polypeptide. Also described are methods engineering cells for high level expression, methods of large scale protein production, and methods for treatment of disease <i>in vivo</i> using viral delivery systems and recombinant cell lines.</p>		

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DESCRIPTION**RECOMBINANT EXPRESSION OF PROTEINS
FROM SECRETORY CELL LINES****I. BACKGROUND OF THE INVENTION****A. Field of the Invention**

The present invention is related to the recombinant expression of proteins from eukaryotic cells. More particularly, the invention relates to the production of recombinant proteins from genetically engineered secretory cells. Methods for use of the cells also are provided.

B. Related Art

Mammalian cells of neuroendocrine origin have been used extensively over the last fifteen years as systems for the study of pathways and mechanisms of polypeptide secretion (Burgess and Kelly, 1987 and Chavez *et al.*, 1994). Examples of cell lines in which such studies have been carried out include the mouse pituitary line AtT-20 (ATCC CCL 89), the rat pituitary growth hormone secreting lines GH3 (ATCC CCL 82.1), the insulin secreting β TC lines derived from transgenic mice expressing SV40 T antigen (Efrat *et al.*, 1988), radiation induced, rat islet cell tumor derived RIN lines (Gazdar, *et al.*, 1980) and the rat adrenal pheochromocytoma cell line PC12 (ATCC CRL 1721). These cell lines maintain many of their endogenous functions, including synthesis of peptide hormones destined for the regulated secretory pathway. These cell lines also are transfectable, allowing expression of novel transgenes for studies of heterologous protein systems.

Three major areas have been studied using these heterologous systems. The first is the study of the sorting mechanism, whereby a given protein, destined for secretion, is targeted to the regulated secretory pathway or the default constitutive secretory pathway.

The second relates to understanding the complex process of secretory protein maturation. This would include the specific steps of protein folding, disulfide bond formation, glycosylation, endoproteolytic processing and post-translational modifications of specific amino acids as well as understanding the enzymes involved in these processes. And the
5 third relates to control of the regulated release of peptide hormones from secretory granules following physiological stimuli.

Neuroendocrine cell lines have been generated in which genes encoding specific peptide hormones have been stably inserted. These enzymes include insulin (Moore *et al.*,
10 1983, Powell *et al.*, 1988 and Gross *et al.*, 1989), somatostatin (Sevarino *et al.*, 1987), thyrotropin-releasing hormone (Sevarino *et al.*, 1989), neuropeptide Y (Dickerson *et al.*, 1987), insulin-like growth factor-1 (Schmidt and Moore, 1994), proopiomelanocortin (Thorne *et al.*, 1989), glucagon (Drucker *et al.*, 1986 and Rouille *et al.*, 1994), pancreatic polypeptide (Takeuchi *et al.*, 1991) and growth hormone (Moore and Kelly, 1985). In
15 general, heterologous expression of these proteins has demonstrated faithful sorting to the regulated secretory pathway, as well as maturation of the proteins in the secretory granules. However, the expression levels of the heterologous proteins have generally been low when compared to normal endogenous expression of the same proteins in a homologous system.

Neuroendocrine cell lines expressing the enzymes involved in the processing of peptide hormones in secretory granules also have been generated. These include the endoproteases PC2 and PC3 (Ohagi *et al.*, 1992, Benjannet *et al.*, 1993, and Rouille *et al.*,
20 1995) and peptidylglycine alpha-amidating monooxygenase (PAM) (Milgram *et al.*, 1992 and Yun and Eipper, 1995). Overexpression of these processing enzymes has helped
25 dissect their relative contributions to peptide hormone processing as well as their intracellular sites of action. These studies demonstrate the academic use of neuroendocrine cells in studying the regulated secretory pathway.

A series of papers over the last five years has addressed the possibility of production of heterologous peptide hormones in neuroendocrine cells. Three of these reports (Sambanis *et al.*, 1990 and 1991, Grampp *et al.*, 1992) use previously established AtT-20 lines expressing either insulin (Moore *et al.*, 1983) or growth hormone (Moore and Kelly, 1985). The highest level of secretion of insulin under stimulated conditions was in the range of 35 to 144 microunits/million cells/hour (equivalent to 1 to 5 ng insulin/million cells/hr). Growth hormone secretion under stimulated conditions was 130 to 340 ng/million cells/hour. These levels of production are well below those reported in the literature for growth hormone production from other recombinant systems (Pavlakakis and Hamer, 1983 and Heartlein *et al.*, 1994). Another study dealing with protein production from a neuroendocrine cell makes use of an insulinoma line engineered to express prolactin (Chen *et al.*, 1995). Absolute levels of production of prolactin on a per cell basis are not reported. A neuroendocrine cell-based system for either *in vitro*, biologically active peptide hormone production or for *in vivo*, cell-based delivery of biologically active peptide hormones has not been achieved in any of these earlier studies.

At least five important features should be addressed in developing a neuroendocrine cell-based system for protein production. The first feature is the absolute level of production of the polypeptide in question. A sufficiently high level of production to make either *in vitro* purification or *in vivo* efficacy must be achieved. As stated above, while many groups have reported expression of recombinant proteins in neuroendocrine lines, the proteins are produced at very low levels.

The second feature is the need for quantitative processing of the peptide to their biologically active forms. Neuroendocrine cell lines maintain variable levels of the enzymes responsible for peptide hormone processing and in many lines the enzyme levels may be insufficient to ensure sufficient processing. This is a critical parameter, especially as attempts are made to engineer high level production of specific peptide hormone transgenes.

The third feature is the need to maintain a dynamic response of the regulated secretory pathway. For both *in vivo* and *in vitro* use of a neuroendocrine cell-based system, the ability to quickly release high concentrations of the biologically active peptide by extracellular stimuli is important. *In vivo* modulation of peptide hormone release is required for titrating the biological efficacy of the cell-based delivery. *In vitro* modulation of peptide hormone release establishes efficient production of highly enriched fractions of starting material for subsequent purification.

The fourth feature is the ability to further engineer other functions into neuroendocrine cells other than just the high-level production of a given polypeptide. This further engineering could involve augmenting the cells capabilities such that any of the three previous points are improved or stabilized (*i.e.*, increased protein levels, increased processing efficiencies or increased dynamic regulated secretory response).

A final engineering maneuver of significance is the ability to reduce or completely ablate the endogenous expression of an unwanted gene product. Reduction or ablation may result in an improved capability to produce, process or release the heterologous polypeptide. Such maneuvers also may confer advantages by removing unwanted or contaminating biological properties of the endogenous peptide hormone. Endogenous peptide production also might counteract the biological activities of the exogenous peptide hormone being produced, resulting in unwanted immunological reactions, reducing the capacity of the engineered lines to quantitatively produce the exogenously engineered protein or complicating purification of the exogenously produced protein. Because all of the existing neuroendocrine cell lines produce endogenous secreted proteins, these concerns are significant.

Thus, despite the benefits of developing a secretory cell line in which the protein synthetic machinery has been commandeered for the production of a heterologous polypeptide, there appear to be significant technical obstacles that are not addressed by the art. As a result, there currently exist no engineered cells that address all of these problems.

II. SUMMARY OF THE INVENTION

The present invention pertains to the engineering of mammalian cells for production of heterologous proteins, for example, in the production of secreted peptide hormones. These mammalian cells also may be engineered such that production of at least one endogenous gene is blocked by molecular engineering, *i.e.*, permitting the usurping of the machinery for the production of the heterologous protein.

Therefore, there is provided a method for producing a polypeptide comprising providing a secretory host cell, blocking the production of an endogenous, secreted polypeptide, contacting with the host cell an exogenous polynucleotide comprising a gene encoding an exogenous polypeptide, wherein the gene is under the control of a promoter active in eukaryotic cells, and culturing the secretory host cell under conditions such that the exogenous polynucleotide expresses the exogenous polypeptide.

In particular embodiments, the promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1. The exogenous polynucleotide may further comprise an adenovirus tripartite 5' leader sequence and intron, and the intron may comprise the 5' donor site of the adenovirus major late transcript and the 3' splice site of an immunoglobulin gene. The exogenous polynucleotide may further comprise a polyadenylation signal.

The secretory host cell may be a neuroendocrine cell, such as an insulinoma, more particularly, a rat insulinoma cell or a human insulinoma cell. It also may be glucose responsive or non-glucose responsive.

The exogenous polypeptide may be secreted, amidated or a fusion protein. Amidated polypeptides include calcitonin, calcitonin gene related peptide (CGRP), β -calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40) (PTH-rP), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related

protein (107-111) (PTH-rP), cholecystokinin (27-33) (CCK), galanin message associated peptide, preprogalanin (65-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamide, metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

The exogenous polypeptide may be a hormone, such as growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I, angiotensin II, β -endorphin, β -melanocyte stimulating hormone (β -MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin. In the case of insulin, recombinant cells having an insulin content of at least about 1000, 1250, 1500 and 2500 ng per 10^6 cells are provided. Recombinant cells producing 200, 300, 400, 500 and 1000 ng of insulin per 10^6 cells per hour also are provided. Recombinant cells secreting at least 25 μ g of human growth hormone per 10^6 cells per hour, at least 50 μ g of human growth hormone per 10^6 cells per hour and about 200 μ g of human growth hormone per 10^6 cells per hour are provided.

The exogenous polypeptide may be a growth factor, such as epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1.

In a particular embodiment, the endogenous, secreted polypeptide and the exogenous polypeptide are the same, for example, where both the endogenous, secreted polypeptide and the exogenous polypeptide are insulin.

In another embodiment, the exogenous polypeptide enhances the production and/or secretion of at least one polypeptide produced by said cell, for example, a protein processing enzyme, a receptor and a transcription factor. Examples include hexokinase, glucokinase, GLUT-2, GLP-1, IPF1, PC2, PC3, PAM, glucagon-like peptide I receptor, glucose-dependent insulintropic polypeptide receptor, BIR, SUR, GHRFR and GHRHR.

Other elements that may be included in the construct are a selectable marker and an internal ribosome entry site.

Methods for blocking of production of an endogenous, secreted polypeptide include expression of an RNA antisense to the DNA or mRNA corresponding to the endogenous, secreted polypeptide, production of ribozyme specific for the mRNA of the endogenous, secreted polypeptide, interruption of the gene encoding said endogenous, secreted polypeptide by homologous recombination, genomic site directed mutagenesis or random integration. As used herein, genomic site directed mutagenesis may employ RNA:DNA oligonucleotides or DNA:DNA oligonucleotides.

Also contemplated by the present invention are large scale production methods including stirring a suspension of the secretory host cell, gas stream agitation of a suspension of the secretory host cell, incubation of the secretory host cell in a non-perfused attached cell container or a perfused attached cell container, culture on microcarriers, microencapsulation of the secretory host cell, followed by cell culture and incubation of the secretory host cell in a perfused packed bed reactor.

Also provided is a method of preventing type I diabetes comprising identifying a subject at risk of type I diabetes and providing to the subject a polynucleotide comprising a human insulin β -chain gene, wherein the β -chain gene is under the control of a promoter active in eukaryotic cells. The providing may comprise introducing the polynucleotide to a cell of the subject *in vivo*. Alternatively, the providing comprises contacting with a

secretory host cell *ex vivo* and administering the secretory host cell to the subject. Further, the expression of the endogenous insulin β -chain in said secretory host cell may be blocked. An advantageous vehicle for providing of the polynucleotide is in a packageable, replication defective adenoviral expression construct.

5

A further embodiment includes a method for treating a subject afflicted with diabetes comprising identifying a subject afflicted with diabetes and providing to the subject a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide has been blocked and (ii) wherein the secretory host cell comprises an
10 exogenous polynucleotide comprising a gene encoding insulin, wherein the gene is under the control of a promoter active in eukaryotic cells.

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In yet another embodiment, there is provided a method for providing a polypeptide to an animal comprising the step of providing to the animal a secretory host cell, wherein
15 (i) the production of an endogenous, secreted polypeptide in the secretory host cell has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding the polypeptide, wherein the gene is under the control of a promoter active in eukaryotic cells.

15

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those
20 skilled in the art from this detailed description.

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III. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better

understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIG. 1: Map of wild-type HKI allele, vector for replacement, and disrupted HKI allele. Arrows indicate the direction of transcription of hexokinase 1 (E1 for exon 1 shown), neomycin resistance (positive selection gene) and the hsv-tk (negative selection gene). Oligos 1, 2, 3 and 4 used in PCRTM analysis are indicated. Capital bold letters indicate restriction enzyme sites introduced by the knock-out vector and lower case letters indicate sites in the endogenous gene. b, B = *Bam*HI; e = *Eco*RI; k = *Kpn*I; N = *Not*I; X = *Xho*I. The 16 kB *Kpn*I fragment cloned from RIN 1046-38 genomic DNA is indicated as well as the probe used in genomic Southern (FIG. 2).

FIG. 2: Genomic Southern confirming hexokinase I gene disruption. The probe (hatched rectangle, Fig. 1) is a 1 kB *Pst* I fragment upstream of the recombination site. Genomic DNA was digested with *Not*I and *Eco*RI. The DNA in each lane is as follows: first lane, RIN 1046-38; second lane, RIN-52/17 containing a randomly integrated HKI replacement vector; and lane 3, RIN-52/17 containing a disrupted allele of the HKI gene (clone 86/X4).

FIG. 3: Rat insulin 1 gene knockout strategy. Map of wild-type RIN insulin I (RINS-I) allele, vector for replacement, and disrupted RINS-I allele. Restriction enzyme sites are shown. Capital bold letters indicate sites introduced by the replacement vector and lower case letters indicate sites in the endogenous gene. b = *Bam*HI; bg = *Bgl*II; N = *Not*I; P = *Pac*I; s = *Spe*I; x, X = *Xho*I. The coding region for RINS-1 gene is indicated by the rectangle with an arrow showing the direction of transcription. The hatched rectangle indicates the sequence used as a

probe in genomic Southern. The arrows, 1 and 2, show the locations of the primers used to amplify genomic DNA specifically recombined at the RINS-1 gene.

5 **FIG. 4A: Insulin content in engineered cell lines.** Immunoreactive insulin was determined from acid extracts prepared from the following cell lines: RIN 1046-38, R5C.I-17, R5C.I-17 chronically treated with 1.0 mM butyrate, and 11/3E9. Values are reported as μg of insulin per million cells.

10 **FIG. 4B: Basal and stimulated insulin secretion from cell lines engineered to produce human insulin.** Secreted immunoreactive insulin was determined from the following cell lines: RIN 1046-38, R5C.I-17, R5C.I-17 chronically treated with 1.0 mM butyrate, and 11/3E9. Basal samples are from a one hour incubation in media lacking glucose and containing 100 μM diazoxide. Stimulated samples are from cells incubated for one hour in media containing 5 mM glucose, 100 μM carbachol, 100 μM IBMX and amino acids. Values are reported as ng of insulin per million cells per hour.

20 **FIG. 5A, FIG. 5B, FIG. 5C: Human proinsulin is efficiently processed to mature insulin.** Immunoreactive insulin was determined from HPLC fractionated acid/ethanol extracts prepared from RIN 1046-38 (FIG. 5A), R5C.I-17 (FIG. 5B) and EP11/3E9 (FIG. 5C). Arrows indicate positions where the following standards elute: mature rat and human insulin (RI and HI), rat and human proinsulin (RPI and HPI), and rat and human processing intermediates des-31,32- and des-64,65-split proinsulin (R 3132, R 6465, H 3132, and H 6465).

FIG. 6A and FIG. 6B: Blood glucose levels of nude rats injected with human insulin-producing cells. Nude rats were injected with either 3 million R5C.I-17 cells (NR1-4, FIG. 6A) or EP11/3E9 cells (NR21-24, FIG. 6B) on day 0. NR5 is an uninjected control animal. Blood glucose was determined on the indicated days. NR1, NR2 and NR23 died prematurely from severe hypoglycemia.

FIG. 7: Insulin message analysis from tumors explanted from nude rats injected with R5C.I-17 cells (see NR1-4, FIG. 6). Primer extension analysis of endogenous rat insulin produces a 91 base extended product (lower band) while the human insulin transgene produces a 101 base extended product (upper band). Analysis of *in vitro* maintained RIN 1046-38 is shown in the first lane and *in vitro* maintained R5C.I-17 is shown in the second and last lanes. The day of tumor explant is indicated for each *in vivo* sample.

FIG. 8: *In vivo* potency of engineered RIN cell lines. The *in vitro* stimulated insulin secretion values of RIN 1046-38, R5C.I-17 and EP11/3E9 (see FIG. 4B) are compared to the explanted tumor mass at initial onset of hypoglycemia in nude rats (see FIG. 6). Individual tumor masses are indicated.

FIG. 9: Gene expression of many endogenous genes is stable *in vitro* versus *in vivo* with the noted exception of GLUT-2. Northern analysis of RNA from *in vitro* maintained cells versus day 25 *in vivo* tumors (R5C.I-17 cells). Signals on Northern blots are running at correct sizes relative to published messages- islet GK - 2.8 kB (Hughes *et al.*, 1991); GAPDH - 1.3 kB (Fort *et al.*, 1985); amylin - 0.9 kB (Leffert *et al.*, 1989); IPF1 - 1.4 kB (Leonard *et al.*, 1993 and Miller *et al.*, 1994); Sulfonylurea receptor - 5.1 kB (Aguilar-Bryan *et al.*, 1995); HK1 - 3.7 kB (Schwab

and Wilson, 1989); GLUT-2 - 2.6 kB (Thorens, *et al.*, 1988); human insulin transgene - 0.7 kB (this study); and Neo transgene - 1.6 kB (this study).

5 **FIG. 10A:** GLUT-2 transgene expression as driven by the CMV promoter is stable *in vitro* and *in vivo*. Northern analysis of GLUT-2 transgene expression of a cell line expressing high levels of GLUT-2 (49/206) is maintained *in vivo* following a 16 or 34 day passage of the insulinoma in a nude rat model.

10 **FIG. 10B:** Low level of endogenous GLUT-2 expression seen in the parental RIN cells maintained *in vitro* (Lane 1, Panels A and B) is lost following a 24 day passage of the cells *in vivo*. The message for GAPDH serves as a loading control.

15 **FIG. 11:** Increased insulin content resulting from expression plasmids containing internal ribosome entry sites (IRES). Immuno reactive insulin was determined from acid/ethanol extracts from 29 independent G418 resistant clones (EP18/3 clones) generated from pCMV8/INS/IRES/NEO.

20 Values are reported as a percentage of the insulin content in R5C.I-17 cells.

25 **FIG. 12A:** Higher human insulin-producing clones generated by iterative engineering of RIN clones with IRES-containing insulin expression plasmids. Northern analysis of EP18/3E1 (FIG. 11), a clone expressing a human insulin/IRES/NEO transgene (first lane) and clones of EP18/3E1 expressing a second transgene encoding human insulin/IRES/Puromycin (EP111/205, 206, 227, and 230). The neomycin containing message is 1.9 kB while the puromycin containing message is 1.7 kB. Messages

30 were detected with a probe specific for human insulin.

FIG. 12B: Increase in insulin content following iterative engineering of RIN clones. Insulin content was determined from acid/ethanol extracts of 18/3E1 cells and 5 clones derived from 18/3E1 expressing a second human insulin transgene (EP111/205, 206, 220, 228 and 230). Cell counts were determined as values are reported as ng insulin per million cells.

FIG. 13: Northern blot analysis of promoter activity in stably transfected RIN lines. Different promoters were driving expression of the common transgene, INS/IRES/NEO, were constructed. For RIP/RIPi, the 5' generic intron from INS/IRES/NEO was replaced with the rat insulin 1 gene intron (RIPi). All lanes contained 10 micrograms of total cellular RNA. The lane labeled RIN38 contains RNA from untransfected cells. The lane labeled PC (PolyClone) contains RNA from a pool of RIN38 clones transfected with pFFE3/RIP8/INS/IRES/NEO.

FIG. 14: Human growth hormone production in RIN cells. Secreted growth hormone was determined from six independent RIN clones. Conditioned media samples were collected from each following a one hour incubation in media lacking glucose and containing 100 μ M diazoxide (Basal/hr), a one hour incubation in media containing 5 mM glucose, 100 μ M carbachol, 100 μ M IBMX and amino acids (Stimulated/hr), and a 24 hr collection in standard tissue culture media containing 11 mM glucose and 5% fetal calf serum. Cell counts were determined as described and values are reported as μ g growth hormone per million cells.

FIG. 15A: Coexpression of PAM and amylin in cell lines. Endogenous levels of expression of PAM and amylin in a series of cell lines was

determined by Northern analysis. Cell lines examined were RIN 1046-38, AtT-20, RIN 1027-B2 and RIN 1046-44 (Phillipe *et al.*, 1987), EP18/3G8 and EP53/114 (this study). Pam message runs at 3.5 to 4.0 kB (Stoffers *et al.*, 1989) while amylin message is 0.9 kB (Leffert *et al.*, 1989)

FIG. 15B: Northern analysis of RIN 1046-38 cells stably transfected with an amylin expression plasmid demonstrates high level expression of the transgene. Amylin is expressed as a amylin/IRES/NEO bicistronic message of 2.1 kB in the polyclone. Endogenous expression of amylin is present in the polyclone as well as RIN 1046-38 and R5C-I.17.

FIG. 16: Insulin Promoter Factor 1 (IPF-1) transgene expression in RIN cells. Levels of stably-transfected IPF-1 mRNA expressed in RIN 38 polyclones and monoclonal cell lines were determined by Northern blot analysis. All lanes contained 10 µg of total cellular RNA. The lane labeled RIN 38 contains RNA from untransfected cells. The lane labeled INS1 contains RNA from another untransfected stable b cell line called INS1. IPF-1 transgene mRNAs is denoted by IPF-1/IRES/NEO. Also shown are levels of endogenous IPF-1 in different RIN lines.

FIG. 17 : Iterative engineering of RIN cells for simultaneous overexpression of human insulin, glucokinase and Glut-2 transgenes. Ten µg of total RNA from parental RIN 1046-38 cells, R5C.I-17 cells expressing human insulin, EP 23/21 cells expressing human insulin and a glucokinase transgene, and EP 49/206 cells expressing human insulin, glucokinase and GLUT-2 transgenes was analyzed and expressed transcripts detected either by primer extension analysis (insulin) or hybridization with the indicated cDNA probes (GLUT-2, glucokinase) as described in Materials and Methods. Insulin primer extension generates

product of 91 bases for endogenous rat insulin and 101 bases for human insulin.

5 **FIG. 18 : Immunoblot analysis of glucokinase expression in RIN cell lines.** Extracts were prepared from RIN 1046-38 cells of low passage (RIN 38), from RIN cells expressing the human proinsulin transgene alone (R5C.I-17), from RIN cells expressing the human proinsulin, GLUT-2, and glucokinase transgenes, with a small increase in glucokinase mRNA (EP 49/206) and from RIN cells expressing the human proinsulin and
10 glucokinase transgenes, with a large increase in glucokinase mRNA (EP 40/110). Glucokinase protein (shown by the arrow and the label "GK") was detected by immunoblotting as described in Materials and Methods, using 5 µg protein/lane. The larger bands of approximately 62-64 kd detected in all lanes are explained by cross-reactivity of the antibody with albumin, based on experiments with pure BSA. The blot shown is
15 representative of three independent experiments.

FIG. 19A and FIG. 19B: Glucose phosphorylation in RIN cell lines. Glucose phosphorylation was measured as described in Materials and
20 Methods in extracts from the indicated cell lines (see legend to FIG. 18 for description of cell lines). **FIG. 19A.** Glucose phosphorylation measured in the absence of glucose-6-phosphate. **FIG. 19B.** Glucose phosphorylation measured in the presence of 10 mM glucose-6-phosphate to inhibit low Km hexokinases. The symbols (*) and (#) indicates those cell lines with
25 glucose phosphorylating activity greater than either of the control cell lines (RIN 38 or R5C.I-17) at a level of significance of $p = 0.003$ and $p < 0.001$, respectively.

FIG. 20 : Basal insulin secretion from RIN cell lines. Basal insulin
30 secretion was measured as described in Materials and Methods from the

indicated cell lines (see legend to FIG. 18 for description of cell lines). Basal insulin secretion was defined as that measured from cells incubated with HBBSS, 0.5% BSA lacking secretagogues for 2 hours. Values represent the mean \pm S.E.M. for 12 determinations. The symbol (#) indicates that R5C.I-17 cells had a higher basal insulin release than the other three lines, with significance $p < 0.001$.

FIG. 21 : Insulin secretion from RIN cell lines in response to glucose and/or IBMX. Insulin secretion was measured from the indicated cell lines in 2 hour static incubation experiments in the presence of the indicated secretagogues. For each cell line, basal insulin secretion in the absence of secretagogues was normalized to 1 (No additives) and secretion in response to secretagogues expressed as fold-increase relative to basal. Values represent the mean \pm S.E.M. for 12 determinations. The symbol (*) indicates that the EP 49/206 and EP 40/110 cells had a larger fold-response to glucose alone or glucose + IBMX than either of the control cell lines, with significance $p < 0.001$.

FIG. 22 : Insulin secretion from perfused RIN cell lines. Cell lines were grown on polyester discs and perfused as described in Materials and Methods. Data are normalized to the average insulin secretion measured in samples 0 through 22 for each cell line, during which cells were being perfused with HBBSS, 0.5% BSA with no added secretagogues. Over the indicated interval, cells were perfused with 10 mM glucose + 100 μ M IBMX (Stimulus). Fractions were collected every two minutes for insulin radioimmunoassay. Cell lines studied were RIN 1046-38 (*), R5C.I-17 (■), and EP 40/110 (○). Data for line EP 49/206 were essentially identical to EP 40/110 and are not shown in the interest of clarity. Data are representative of 1-2 independent experiments for each cell line.

FIG. 23A, FIG. 23B, FIG. 23C and FIG.23D: Glucose dose-response profile of RIN cell lines in the presence and absence of 5-thiogluucose.

Insulin secretion was measured from the indicated cell lines in 2 hour static incubation experiments in the presence of the indicated secretagogues.

Studies at 0 mM glucose were carried out in the presence or absence of 100 μ M IBMX. The symbol (+) following the glucose concentration on the x-axis indicates that 100 μ M IBMX was present in all other assays. The assays were also carried out in the absence of 5-thiogluucose (open bars) or in the presence of 2 mM 5-thiogluucose (darkened bars). FIG. 23A, RIN 1046-38 cells; FIG. 23B R5C.I-17 cells, FIG. 23C, EP 49/206 cells; FIG.23D, EP 40/110 cells. Each bar represents the mean \pm S.D. for 3 independent determinations. Note the difference in scale (y-axis) for each panel.

FIG. 24 : 5-³H glucose usage in RIN cell lines. 5-³H glucose usage was measured in the indicated cell lines as described in Materials and Methods. Data represent the mean \pm S.D. for 3 independent determinations.

IV. DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Secretory cells, especially neuroendocrine cells, have several endogenous functions that make them uniquely suited for production of a wide range of proteins, including secreted peptide hormones. These specialized functions include the regulated secretory pathway. The regulated secretory pathway embodies the secretory granules of neuroendocrine cells which serve as the site of maturation and storage of a large class of peptide hormones with profound biological functions. Proper biological function of the peptides is due both to their secretion in a regulated and titratable manner as well as a complex set of post-translational modifications resulting in the final biologically active product. As a result, these cells can be used *in vitro* to produce large amounts of proteins, *in vivo* to supply therapeutic proteins, or *in vivo* to immunize hosts, for example, in the production of monoclonal antibodies.

The present invention is designed to take advantage of this secretory machinery for the purpose of producing heterologous proteins. A variety of different modifications may be made to increase the efficiency of the cell, one possible modification is the blocking of production of an endogenous protein in the host cell. This will, in essence, "make room" for the heterologous protein and, hence, avoid competition between the endogenous and heterologous proteins during synthesis. The components for such a system, and methods of producing proteins therewith, are set forth in detail below.

A. Host Cells

Engineering of secretory cells to synthesize proteins for either *in vitro* large scale production, or for *in vivo* cell-based delivery, will advantageously make use of many attributes of these cells. Regulated secretory cells present a natural bioreactor containing specialized enzymes involved in the processing and maturation of secreted proteins. These processing enzymes include endoproteases (Steiner *et al.*, 1992) and carboxypeptidases (Fricker, 1988) for the cleavage of prohormones to hormones and PAM, an enzyme catalyzing the amidation of a number of peptide hormones (Eipper *et al.*, 1992a). Similarly, maturation and folding of peptide hormones is performed in a controlled, stepwise manner with defined parameters including pH, calcium and redox states.

Complete processing requires sufficient levels of the processing enzymes as well as sufficient retention of the maturing peptides. In this way, physiological signals leading to the release of the contents of the secretory granules ensures release of fully processed, active proteins. This is important for both maximum production for *in vitro* purposes and for the possible use of cells for *in vivo* purposes.

All cells secrete proteins through a constitutive, non-regulated secretory pathway. A subset of cells are able to secrete proteins through a specialized regulated secretory pathway. Proteins destined for secretion by either mechanism are targeted to the endoplasmic reticulum and pass through the golgi apparatus. Constitutively secreted

proteins pass directly from the golgi to the plasma membrane in vesicles, fusing and releasing the contents constitutively without the need for external stimuli. In cells with a regulated pathway, proteins leave the golgi and concentrate in storage vesicles or secretory granules. Release of the proteins from secretory granules is regulated, requiring an external stimuli. This external stimuli, defined as a secretagogue, can vary depending on cell type, optimal concentration of secretagogue, and dynamics of secretion. Proteins can be stored in secretory granules in their final processed form for long periods of time. In this way a large intracellular pool of mature secretory product exists which can be released quickly upon secretagogue stimulation.

A cell specialized for secreting proteins via a regulated pathway can also secrete proteins via the constitutive secretory pathway. Many cell types secrete proteins by the constitutive pathway with little or no secretion through a regulated pathway. As used herein, "secretory cell" defines cells specialized for regulated secretion, and excludes cells that are not specialized for regulated secretion. The regulated secretory pathway is found in secretory cell types such as endocrine, exocrine, neuronal, some gastrointestinal tract cells and other cells of the diffuse endocrine system.

(i) Glucose Responsive Cells.

For delivery of some peptide hormones or factors, it may be desirable to cause the polypeptide to be released from cells in response to changes in the circulating glucose concentration. The most obvious example of a secretory cell type that is regulated in this fashion is the β -cell of the pancreatic islets of Langerhans, which releases insulin in response to changes in the blood glucose concentration. Engineering of primary β -cells for production of products other than insulin is not practical. Instead, a preferred vehicle may be one of the several cell lines derived from islet β -cells that have emerged over the past two decades. While early lines were derived from radiation- or virus-induced tumors (Gazdar *et al.*, 1980, Santerre *et al.*, 1981), more recent work has centered on the application of transgenic technology (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990). A general approach taken with the latter technique is to express an oncogene, most often SV40 T-

antigen, under control of the insulin promoter in transgenic animals, thereby generating β -cell tumors that can be used for propagating insulinoma cell lines (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990). While insulinoma lines provide an advantage in that they can be grown in essentially unlimited quantity at relatively low cost, most exhibit differences in their glucose-stimulated insulin secretory response relative to normal islets. These differences can be quite profound, such as in the case of RINm5F cells, which were derived from a radiation-induced insulinoma and which in their current form are completely lacking in any acute glucose-stimulated insulin secretion response (Halban *et al.*, 1983, Shimuzu *et al.*, 1988). RIN 1046-38 cells are also derived from a radiation-induced insulinoma but can be shown to be glucose responsive when studied at low passage numbers (Clark *et al.*, 1990). This response is maximal at subphysiological glucose concentrations and is lost entirely when these cells are cultured for more than 40 passages (Clark *et al.*, 1990). GLUT-2 and glucokinase are expressed in low passage RIN 1046-38 cells but are gradually diminished with time in culture in synchrony with the loss of glucose-stimulated insulin release (Ferber *et al.*, 1994). Restoration of GLUT-2 and glucokinase expression in RIN 1046-38 cells by stable transfection restores glucose-stimulated insulin secretion (Ferber *et al.*, 1994), and the use of these genes as a general tool for engineering of glucose sensing has been described in a previously issued patent (Newgard, US Patent 5,427,940). RIN 1046-38 cells transfected with the GLUT-2 gene alone are maximally glucose responsive at low concentrations of the sugar (approximately 50 μ M), but the threshold for response can be shifted by preincubating the cells with 2-deoxyglucose, which when converted to 2-deoxyglucose-6-phosphate inside the cell serves as an inhibitor of low K_m hexokinase, but not glucose activity (Ferber *et al.*, 1994).

Recently, Asafari *et al.* have reported on the isolation of a new insulinoma cell line called INS-1 that retains many of the characteristics of the differentiated β -cell, most notably a relatively high insulin content and a glucose-stimulated insulin secretion response that occurs over the physiological range (Asafari *et al.*, 1992). This line was isolated by propagating cells freshly dispersed from an X-ray induced insulinoma tumor in media containing 2-mercaptoethanol. Consistent with the finding of physiological glucose

responsiveness. a recent report indicates that INS-1 cells express GLUT-2 and glucokinase as their predominant glucose transporter and glucose phosphorylating enzyme, respectively (Marie *et al.*, 1993). INS-1 cells grow very slowly and require 2-mercaptoethanol. It remains to be determined whether glucose responsiveness and expression of GLUT-2 and glucokinase are retained with prolonged culturing of these cells.

Cell lines derived by transgenic expression of T-antigen in β -cells (generally termed β TC cells) also exhibit variable phenotypes (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990, Whitesell *et al.*, 1991 and Efrat *et al.*, 1993). Some lines have little glucose-stimulated insulin release or exhibit maximal responses at subphysiological glucose concentrations (Efrat *et al.*, 1988, Miyazaki *et al.*, 1990, Whitesell *et al.*, 1991), while others respond to glucose concentrations over the physiological range (Miyazaki *et al.*, 1990 and Efrat *et al.*, 1993). It appears that the near-normal responsiveness of the latter cell lines is not permanent, since further time in culture results in a shift in glucose dose response such that the cells secrete insulin at subphysiological glucose concentrations (Efrat *et al.*, 1993). In some cases, these changes have been correlated with changes in the expression of glucose transporters and glucose-phosphorylating enzymes. Miyazaki *et al.* isolated two classes of clones from transgenic animals expressing an insulin promoter/T-antigen construct. Glucose-unresponsive lines such as MIN-7 were found to express GLUT-1 rather than GLUT-2 as their major glucose transporter isoform, while MIN-6 cells were found to express GLUT-2 and to exhibit normal glucose-stimulated insulin secretion (Miyazaki *et al.*, 1990). More recently, Efrat and coworkers demonstrated that their cell line bTC-6, which exhibits a glucose-stimulated insulin secretion response that resembles that of the islet in magnitude and concentration dependence, expressed GLUT-2 and contained a glucokinase:hexokinase activity ratio similar to that of the normal islet (Efrat *et al.*, 1993). With time in culture, glucose-stimulated insulin release became maximal at low, subphysiological glucose concentrations. GLUT-2 expression did not change with time in culture, and glucokinase activity actually increased slightly, but the major change was a large (approximately 6-fold) increase in hexokinase expression (Efrat

et al., 1993). Furthermore, overexpression of hexokinase I, but not GLUT-1, in well-differentiated MIN-6 cells results in both increased glucose metabolism and insulin release at subphysiological glucose concentrations. Similar results have been obtained upon overexpression of hexokinase I in normal rat islets (Becker *et al.*, 1994b). These results are all consistent with the observations of Ferber, *et al.* described above in showing that a high hexokinase:glucokinase ratio will cause insulin-secreting cells to respond to glucose concentrations less than those required to stimulate the normal β -cell.

(ii) Non-glucose Responsive Cells

An alternative to insulinoma cell lines are non-islet cell lines of neuroendocrine origin that are engineered for insulin expression. The foremost example of this is the AtT-20 cell, which is derived from ACTH secreting cells of the anterior pituitary. A decade ago, Moore *et al.* demonstrated that stable transfection of AtT-20 cells with a construct in which a viral promoter is used to direct expression of the human proinsulin cDNA resulted in cell lines that secreted the correctly processed and mature insulin polypeptide (Moore *et al.*, 1983). Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin. This suggests that these cells contain a regulated secretory pathway that is similar to that operative in the islet β -cell (Moore *et al.*, 1983, Gross *et al.*, 1989). More recently, it has become clear that the endopeptidases that process proinsulin to insulin in the islet β -cell, termed PC2 and PC3, are also expressed in AtT-20ins cells (Smeekens *et al.*, 1990, Hakes *et al.*, 1991). AtT-20ins cells do not respond to glucose as a secretagogue (Hughes *et al.*, 1991). Interestingly, AtT-20 cells express the glucokinase gene (Hughes *et al.*, 1991, Liang *et al.*, 1991) and at least in some lines, low levels of glucokinase activity (Hughes *et al.*, 1991 and 1992, Quaade *et al.*, 1991), but are completely lacking in GLUT-2 expression (Hughes *et al.*, 1991 and 1992). Stable transfection of these cells with GLUT-2, but not the related transporter GLUT-1, confers glucose-stimulated insulin secretion, albeit with maximal responsiveness at subphysiological glucose levels, probably because of a non-optimal hexokinase:glucokinase ratio (Hughes *et al.*, 1992, 1993).

The studies with AtT-20ins cells are important because they demonstrate that neuroendocrine cell lines that normally lack glucose-stimulated peptide release may be engineered for this function. Other cell lines that are characterized as neuroendocrine, but lacking in endogenous glucose response include PC12, a neuronal cell line (ATCC CRL 1721) and GH3, an anterior pituitary cell line that secretes growth hormone (ATCC CCL82.1). It is not possible to determine whether such cell lines will gain glucose responsiveness by engineering similar to that described for the AtT-20ins cell system without performing the experiments. However, these lines do exhibit other properties important for this invention such as a regulated secretory pathway, expression of endopeptidases required for processing of prohormones to their mature hormone products, and post-translational modification enzymes. In sum, all neuroendocrine cell lines are useful for the essential aspect of this invention, which is the production of heterologous products in a cell line in which the natural product (insulin, growth hormone, ACTH, *etc.*) has been eliminated. Some or all of these lines will also be useful for glucose-regulated product delivery, using the methods described in U.S. Patent 5,427,940 to generate such responsiveness.

(iii) Methods for Blocking Endogenous Protein Production

Blocking expression of an endogenous gene product is an important modification of host cells according to the present invention. The targeted endogenous gene encodes a protein normally secreted by the host cell. Blocking expression of this endogenous gene product, while engineering high level expression of genes of interest, represents a unique way of designing cells for protein production.

Cells generated by this two-step process express heterologous proteins, including a variety of natural or engineered proteins (fusions, chimeras, protein fragments, *etc.*). Cell lines developed in this way are uniquely suited for *in vivo* cell-based delivery or *in vitro* large-scale production of defined peptide hormones with little or no contaminating or unwanted endogenous protein production.

A number of basic approaches are contemplated for blocking of expression of an endogenous gene in host cells. First, constructs are designed to homologously recombine into particular endogenous gene loci, rendering the endogenous gene nonfunctional.

5 Second, constructs are designed to randomly integrate throughout the genome, resulting in loss of expression of the endogenous gene. Third, constructs are designed to introduce nucleic acids complementary to a target endogenous gene. Expression of RNAs corresponding to these complementary nucleic acids will interfere with the transcription and/or translation of the target sequences. Fourth, constructs are designed to introduce
10 nucleic acids encoding ribozymes - RNA-cleaving enzymes - that will specifically cleave a target mRNA corresponding to the endogenous gene. Fifth, endogenous gene can be rendered dysfunctional by genomic site directed mutagenesis

Antisense. Antisense methodology takes advantage of the fact that nucleic acids
15 tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil
20 (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix
25 formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*,
30 such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the

remaining portion of the construct and, therefore, would be used for the rest of the sequence.

Ribozymes. Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor.

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion.

Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cook, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cook *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992).

This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cook *et al.*, 1981). For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Homologous Recombination. Another approach for blocking of endogenous protein production involves the use of homologous recombination. Homologous recombination relies, like antisense, on the tendency of nucleic acids to base pair with

complementary sequences. In this instance, the base pairing serves to facilitate the interaction of two separate nucleic acid molecules so that strand breakage and repair can take place. In other words, the "homologous" aspect of the method relies on sequence homology to bring two complementary sequences into close proximity, while the
5 "recombination" aspect provides for one complementary sequence to replace the other by virtue of the breaking of certain bonds and the formation of others.

Put into practice, homologous recombination is used as follows. First, a target gene is selected within the host cell. Sequences homologous to the target gene are then
10 included in a genetic construct, along with some mutation that will render the target gene inactive (stop codon, interruption, *etc.*). The homologous sequences flanking the inactivating mutation are said to "flank" the mutation. Flanking, in this context, simply means that target homologous sequences are located both upstream (5') and downstream (3') of the mutation. These sequences should correspond to some sequences upstream and
15 downstream of the target gene. The construct is then introduced into the cell, thus permitting recombination between the cellular sequences and the construct.

As a practical matter, the genetic construct will normally act as far more than a vehicle to interrupt the gene. For example, it is important to be able to select for
20 recombinants and, therefore, it is common to include within the construct a selectable marker gene. This gene permits selection of cells that have integrated the construct into their genomic DNA by conferring resistance to various biostatic and biocidal drugs. In addition, a heterologous gene that is to be expressed in the cell also may advantageously be included within the construct. The arrangement might be as follows:

25

...vector•5'-flanking sequence•heterologous gene• selectable marker gene•flanking
sequence-3'•vector...

Thus, using this kind of construct, it is possible, in a single recombinatorial event, to (i) "knock out" an endogenous gene, (ii) provide a selectable marker for identifying such an event and (iii) introduce a heterologous gene for expression.

5 Another refinement of the homologous recombination approach involves the use of a "negative" selectable marker. This marker, unlike the selectable marker, causes death of cells which express the marker. Thus, it is used to identify undesirable recombination events. When seeking to select homologous recombinants using a selectable marker, it is difficult in the initial screening step to identify proper homologous recombinants from
10 recombinants generated from random, non-sequence specific events. These recombinants also may contain the selectable marker gene and may express the heterologous protein of interest, but will, in all likelihood, not have the desired "knock out" phenotype. By attaching a negative selectable marker to the construct, but outside of the flanking regions, one can select against many random recombination events that will incorporate the
15 negative selectable marker. Homologous recombination should not introduce the negative selectable marker, as it is outside of the flanking sequences.

In a particular aspect of this embodiment, the negative selectable maker is GLUT-2. It is also contemplated that GLUT-5 would function in a similar manner to GLUT-2.
20 Therefore, the selection protocols described are intended to refer to the use of both GLUT-2 and GLUT-5.

In a first embodiment, a target gene within a GLUT-2⁺ host cell is selected as the location into which a selected gene is to be transferred. Sequences homologous to the
25 target gene are included in the expression vector, and the selected gene is inserted into the vector such that target gene homologous sequences are interrupted by the selected gene or, put another way, such the target gene homologous sequences "flank" the selected gene. In preferred embodiments, a drug selectable marker gene also is inserted into the target gene homologous sequences. Given this possibility, it should be apparent that the term "flank"
30 is used broadly herein, namely, as describing target homologous sequences that are both

upstream (5') and downstream (3') of the selected gene and/or the drug selectable marker gene. In effect, the flanking sequences need not directly abut the genes they "flank."

The construct for use in this embodiment is further characterized as having a functional GLUT-2 gene attached thereto. Thus, one possible arrangement of sequences would be:

... 5'-GLUT-2•flanking target sequences•selected gene•drug-selectable marker gene•flanking target sequences-3' ...

Of course, the GLUT-2 could come at the 3'-end of the construct and the selected gene and drug-selectable marker genes could exchange positions.

Application of a drug to such cells will permit isolation of recombinants, but further application of Streptozotocin (glucopyranose, 2-deoxy-2-[3-methyl-5-nitrosourido-D]; STZ) to such cells will result in killing of non-homologous recombinants because the incorporated GLUT-2 gene will produce GLUT-2 transporter, rendering the cells susceptible to STZ treatment (the original cell was GLUT-2⁻).

On the other hand, site-specific recombination, relying on the homology between the vector and the target gene, will result in incorporation of the selected gene and the drug selectable marker gene only; GLUT-2 sequences will not be introduced in the homologous recombination event because they lie outside the flanking sequences. These cells will be drug resistant and but not acquire the GLUT-2 sequences and, thus, remain insensitive to STZ. This double-selection procedure (drug^{res}/STZ^{res}) should yield recombinants that lack the target gene and express the selected gene. Further screens for these phenotypes, either functional or immunologic, may be applied.

A modification of this procedure is one where no selected gene is included, *i.e.*, only the selectable marker is inserted into the target gene homologous sequences. Use of

this kind of construct will result in the "knock-out" of the target gene only. Again, proper recombinants are screened by drug resistance and STZ resistance (the original cell was GLUT-2').

5 **Genomic Site-Directed Mutagenesis with Oligonucleotides.** Through analysis of radiation-sensitive mutants of *Ustilago maydis*, genes have been characterized that participate in DNA repair (Tsukuda *et al.*, 1989; Bauchwitz and Holloman, 1990). One such gene, *REC2*, encodes a protein that catalyzes homologous pairing between complementary nucleic acids and is required for a functional recombinational repair
10 pathway (Kmiec *et al.*, 1994; Rubin *et al.*, 1994). *In vitro* characterization of the REC2 protein showed that homologous pairing was more efficient between RNA-DNA hybrids than the corresponding DNA duplexes (Kmiec *et al.*, 1994; PCT, WO 96/22364). However, efficiency in pairing between DNA:DNA duplexes could be enhanced by increasing the length of the DNA oligonucleotides (Kmiec *et al.*, 1994). These
15 observations led investigators to test the use of chimeric RNA-DNA oligonucleotides (RDOs) in the targeted modification of genes in mammalian cell lines (Yoon *et al.*, 1996; Cole-Strauss *et al.*, 1996; PCT WO95/15972). The RNA-DNA oligonucleotides that were used to test this application contained self-annealing sequences such that double-hairpin capped ends are formed. This feature is thought to increase the *in vivo* half-life of the
20 RDO by decreasing degradation by helicases and exonucleases. Further, the RDOs contained a single base pair that differs from the target sequence and otherwise aligns in perfect register. It is believed that the single mismatch will be recognized the DNA repair enzymes. And the RDOs contained RNA residues modified by 2'-O-methylation of the ribose sugar. Such modification makes the RDO resistant to degradation by ribonuclease
25 activity (Monia *et al.*, 1993).

Two separate experimental systems have been used to test the use of RDOs for targeted gene disruption in mammalian cell lines. In one system RDOs were used to target and correct an alkaline phosphatase cDNA in that was maintained in the episomal DNA of
30 Chinese hamster ovary cells. An inactive form of alkaline phosphatase was converted to a

wild-type form with an efficiency of about 30% (Yoon *et al.*, 1996). In a second system, a genetic mutation within chromosomal DNA was targeted and corrected. A lymphoid blast cell line was derived from a patient with sickle cell disease who was homozygous for a point mutation in the β -globin gene. Here again the overall frequency of gene conversion from the mutant to the wild-type form was very high and was found to be dose-dependent on the concentration of the RDOs (Cole-Strauss *et al.*, 1996).

If the use of RDOs or DNA oligonucleotides for the purposes of targeted gene conversion is broadly applicable to various mammalian cell lines, then it offers several advantages to current technologies that have been used to accomplish gene disruption such as homologous recombination. First, if gene conversion by RDO or DNA oligonucleotides occurs in various cell lines at an efficiency of 30% then this will represent a much higher rate than has been reported for targeted gene disruption via homologous recombination. Secondly, only short sequences are required for gene disruption by RDOs or DNA oligonucleotides (typically 60mers to 70mers); whereas homologous recombination requires very long stretches of complementary sequences. Homologous sequences from 9 to 15 kilobases are typically recommended in the construction of targeting vectors. As a result, construction of DNA vectors for homologous recombination usually involves extensive gene mapping studies and time consuming efforts in the isolation of genomic DNA sequences. Such efforts are unnecessary if RDOs are used for targeted gene conversions. Thirdly, assays for gene conversion by RDOs can be performed 4 to 6 hours following introduction of the RDOs or DNA oligonucleotides into the cell. In contrast, gene conversion by homologous recombination requires a relatively long period of time (days to weeks) between the time of introducing the targeting vector DNA and assaying for recombinants.

Random Integration. Though lacking the specificity of homologous recombination, there may be situations where random integration will be used as a method of knocking out a particular endogenous gene. Unlike homologous recombination, the recombinatorial event here is completely random, *i.e.*, not reliant upon base-pairing of

complementary nucleic acid sequences. Random integration is like homologous recombination, however, in that a gene construct, often containing a heterologous gene and a selectable marker, integrates into the target cell genomic DNA via strand breakage and reformation.

5

Because of the lack of sequence specificity, the chances of any given recombinant integrating into the target gene are greatly reduced. Also possible is integration into a second loci, resulting in the loss of expression of the gene of interest. This second locus could encode a transcription factor needed for expression of the first gene, a locus control region needed for the expression of the first gene, *etc.* As a result, it may be necessary to "brute force" the selection process. In other words, it may be necessary to screen hundreds of thousands of drug-resistant recombinants before a desired mutant is found. Screening can be facilitated, for example, by examining recombinants for expression of the target gene using immunologic or even functional tests; expression of the target gene indicate recombination elsewhere and, thus, lack of suitability.

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(iv) Methods for Increasing Production of Recombinant Peptides from Secretory Cells

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The present invention also contemplates augmenting or increasing the capabilities of cells to produce biologically active polypeptides. This can be accomplished, in some instances, by overexpressing the proteins involved in protein processing, such as the endoproteases PC2 and PC3 (Steiner *et al.*, 1992) or the peptide amidating enzyme, PAM (Eipper *et al.*, 1992a) in the case of amidated peptide hormones.

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Expression of proteins involved in maintaining the specialized phenotype of host cells, especially their secretory capacity, is important. Engineering the overexpression of a cell type-specific transcription factor such as the Insulin Promoter Factor 1 (IPF1) found in pancreatic β -cells (Ohlsson *et al.*, 1993) could increase or stabilize the capabilities of engineered neuroendocrine cells. Insulin promoter factor 1 (IPF-1; also referred to as STF-1, IDX-1, PDX-1 and β TF-1) is a homeodomain-containing transcription factor

proposed to play an important role in both pancreatic development and insulin gene expression in mature β cells (Ohlsson *et al.*, 1993, Leonard *et al.*, 1993, Miller *et al.*, 1994, Kruse *et al.*, 1993). In embryos, IPF-1 is expressed prior to islet cell hormone gene expression and is restricted to positions within the primitive foregut where pancreas will later form. Indeed, mice in which the IPF-1 gene is disrupted by targeted knockout do not form a pancreas (Jonsson *et al.*, 1994). Later in pancreatic development, as the different cell types of the pancreas start to emerge, IPF-1 expression becomes restricted predominantly to β cells. IPF-1 binds to TAAT consensus motifs contained within the FLAT E and P1 elements of the insulin enhancer/promoter, whereupon, it interacts with other transcription factors to activate insulin gene transcription (Peers *et al.*, 1994).

Stable overexpression of IPF-1 in neuroendocrine β cell lines will serve two purposes. First, it will increase transgene expression under the control of the insulin enhancer/promoter. Second, because IPF-1 appears to be critically involved in β cell maturation, stable overexpression of IPF-1 in β cell lines should cause these mostly dedifferentiated β -cells to regain the more differentiated function of a normal animal β cell. If so, then these redifferentiated β cell lines could potentially function as a more effective neuroendocrine cell type for cell-based delivery of fully processed, bioactive peptide hormones.

Also, further engineering of cells to generate a more physiologically-relevant regulated secretory response is claimed. Examples would include engineering the ratios of glucokinase to hexokinase in rat insulinoma cells that also overexpress the Type II glucose transporter (GLUT-2) such that a physiologically-relevant glucose-stimulated secretion of peptide hormones is achieved. Other examples include engineering overexpression of other signaling proteins known to play a role in the regulated secretory response of neuroendocrine cells. These include cell surface proteins such as the β -cell-specific inwardly rectifying potassium channel (BIR; Inagaki *et al.*, 1995), involved in release of the secretory granule contents upon glucose stimulation, the sulfonylurea receptor (SUR), and ATP sensitive channel. Other cell surface signaling receptors which help potentiate

the glucose-stimulated degranulation of β -cells including the glucagon-like peptide I receptor (Thorens, 1992) and the glucose-dependent insulinotropic polypeptide receptor (also known as gastric inhibitory peptide receptor) (Usdin, 1993) can be engineered into neuroendocrine cells. These β -cell-specific signaling receptors, as well as GLUT-2 and glucokinase, are involved in secretory granule release in response to glucose. In this way, glucose stimulated release of any heterologous peptide targeted to the secretory granule can be engineered. Alternatively, other cell surface signaling proteins involved in non-glucose-stimulated release of secretory granule contents can be engineered into neuroendocrine cells. Examples would include releasing factor receptors such as Growth Hormone Releasing Factor Receptor (Lin *et al.*, 1992) and Somatostatin or Growth Hormone Releasing Hormone Receptor (Mayo, 1992).

One potential target for genetic engineering to improve cell characteristics for protein production is hexokinase I. It now has been determined that interfering with hexokinase I function reduces the growth rate of cells. The following is a discussion of engineering of hexokinases according to the present invention.

Mitochondrial Binding. Low K_m hexokinases are distinguished from glucokinase in that they are allosterically regulated by glucose-6-phosphate and by binding to mitochondria (Wilson, 1968; 1973; 1985; 1995). Micromolar concentrations of glucose-6-phosphate inhibit the activities of hexokinases I, II, and III, but appreciable inhibition of glucokinase requires glucose-6-phosphate concentrations in excess of 10 mM. Binding of hexokinases I and II to mitochondria alters their kinetic properties (Wilson, 1968; 1985; 1995), while glucokinase does not appear to be capable of binding to mitochondria at all (Becker *et al.* 1996).

When bound to mitochondria, hexokinase I undergoes an increase in affinity (a decrease in K_m) for its substrate ATP (Wilson, 1985). In addition, the enzyme becomes far less inhibitable by glucose-6-phosphate, as indicated by a several-fold increase in K_i for this ligand (Wilson, 1985). Studies with hexokinase I have revealed the existence of two

types of mitochondrial binding sites (Kabeer and Wilson, 1994). Glucose-6-phosphate causes displacement of a proportion of mitochondrially-bound hexokinase from one type of site. The enzyme that remains bound to mitochondria after glucose-6-phosphate treatment is considered to occupy the second site, from which it can be removed by treatment with 0.5 M KSCN.

It has been known for some time that limited digestion of hexokinase I with chymotrypsin yields an enzyme fragment that retains catalytic activity but that loses its capacity for mitochondrial binding, and that enzyme treated in this manner is lacking in a portion of its N-terminal domain (Polakis and Wilson, 1985). The N-terminal sequences of both hexokinases I and II are relatively hydrophobic, and it has been shown that the hydrophobic N-terminus of hexokinase I is capable of insertion into the lipid bilayer of the mitochondrial membrane (Xie and Wilson, 1988).

Subsequently, Gelb *et al.*, (1992) demonstrated that a chimeric protein consisting of the N-terminal 15 amino acids of hexokinase I fused to chloramphenicol acetyltransferase was capable of binding to rat liver mitochondria, and that this binding was competitive with authentic hexokinase I (Gelb *et al.* 1992). Although Gelb *et al.* (1992) have suggested that the first 15 amino acids of hexokinase are sufficient to target such a chimeric protein to mitochondria, these studies were not designed to attempt to alter metabolic regulation in target cell lines. Thus, the elements required to effect displacement of endogenous hexokinase from its mitochondrial binding site were not unequivocally identified in the study of Gelb and co-authors as discussed below.

While the results of Gelb *et al.* (1992) argue for the importance of this small N-terminal segment in targeting of hexokinase to mitochondria, others have suggested that other regions of the molecule may also be important in stabilizing the interaction (Polakis and Wilson, 1985; Felgner and Wilson, 1977; Smith and Wilson, 1991). This is based on studies showing that hexokinase I binding to mitochondria is stabilized by Mg^{2+} , an effect likely reflecting electrostatic interactions between the enzyme and the outer mitochondrial

membrane (*i.e.*, not involving the N-terminal 15 amino acids that are intercalated into the membrane). Therefore, the mitochondrial binding regions of HK have not been clearly identified to date, and there is even less information available on the issue of HK displacement.

5

At least part of hexokinase binding to mitochondria is via interactions with members of a family of proteins known as voltage-dependent anion channels (VDAC) or porins (Fiek *et al.*, 1982; Linden *et al.*, 1982). These porins form a channel through which metabolites such as ATP and various anions traverse the outer mitochondrial membrane. Binding of hexokinases to porin thus may ensure a supply of intramitochondrially-generated ATP as substrate.

10

15

Constructs of the present invention may comprise the N-terminal 15 amino acids of a hexokinase enzyme, preferably hexokinase I or II, since this segment should be easily expressed in cells and retained as a stable peptide. Constructs comprising the entire N-terminal domain of either hexokinase I or hexokinase II, or the intact, full-length hexokinase I or II proteins that have been rendered inactive by site-directed mutagenesis of amino acids that are important for the enzyme's catalytic function are also contemplated. Constructs based upon hexokinase I will be particularly, or even exclusively, preferred in certain embodiments.

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The reason for preferring the N-terminal domain construct is that this element seems to comprise a complete structural domain, based upon studies in which this domain can be expressed in bacteria and shown to bind glucose-6-phosphate (Wilson, 1994; Arora *et al.*, 1993; White and Wilson, 1987; White and Wilson, 1990). This suggests that the intact N-terminal domain should fold and form a structure analogous to its structure in the full-length hexokinase I or II protein. As the present inventors contemplate that this structure mediates attachment of the intact hexokinase protein to mitochondria, the intact, correctly folded N-terminal domain is a preferred embodiment of this invention.

30

For embodiments involving the N-terminal domain, a segment comprising amino acids 1-455 is preferred because of a naturally occurring *NcoI* restriction enzyme site in the DNA sequence corresponding to amino acid 482. This *NcoI* site allows the fragment encoding the N-terminal domain to be easily isolated and subcloned, and also allows direct fusion of the N-terminal domain of hexokinase to the intact functional sequence of glucokinase via an *NcoI* site located at the AUG start codon of this gene.

Of course, it will be understood that peptides, polypeptides and protein domains of any intermediate length between about 15 amino acids and about 455 amino acids, and longer proteins, may be used in displacing endogenous hexokinase from the mitochondria. Accordingly, constructs comprising about 20, about 50, about 100, about 150, about 200, about 300 or about 400 amino acids in length may be used for these purposes. It is also contemplated that an intact hexokinase protein that is rendered catalytically inactive will interact with mitochondria in a manner identical to the active proteins. Expression of such a HK variant is therefore another method for inhibiting endogenous HK (Bajjal and Wilson, 1992). Inactivated, hexokinase proteins include those that have been subjected to chemical mutagenesis and also those produced using molecular biological techniques and recombinant protein production.

The identification of appropriate polypeptide regions and/or particular amino acid sites that may be targeted in order to inactivate hexokinase will be known to those of skill in the art. The crystal structure of certain hexokinase enzymes is available. Coupling the crystal structure information with a comparison of the primary sequence information for various distinct hexokinases will allow one to identify those regions and sites that are important for hexokinase activity, such as the binding sites for ATP, glucose and glucose-6-phosphate. This has been discussed in detail in various publications, such as Printz *et al.* (1993), incorporated herein by reference, which information can be used in connection with preparing mutants and variants for use herewith. Deletion of certain amino acids or peptide segments, as may be achieved by molecular biological manipulation, is another contemplated method for preparing inactive hexokinases.

The enzyme glycerol kinase is another protein thought to bind to mitochondria via porins or VDACs (Adams *et al.*, 1991). Glycerol kinase catalyzes formation of glycerol phosphate from glycerol, using ATP as phosphate donor. Thus, expression of glycerol kinase in cell lines represents an alternative to expression of inactive hexokinase proteins or fragments thereof which is also contemplated for use in the displacement of endogenous low- K_m hexokinases from their normal mitochondrial binding site.

A particularly powerful method of inhibiting hexokinase within a mammalian cell involves the displacement of hexokinase from the mitochondria and the concomitant provision of active glucokinase. This is advantageously achieved by providing to the cell a hexokinase-glucokinase chimera or fusion protein, in which the hexokinase portion is capable of binding to the mitochondria and yet does not exhibit hexokinase catalytic activity, and in which the glucokinase portion is catalytically active. Chemically-fused polypeptides are a possibility, but recombinant proteins are naturally most preferred for use in this manner. The identification of appropriate hexokinase fragments for use in such a chimera has been described herein above.

In terms of the glucokinase portions of these fusion proteins, any glucokinase-derived sequence that contains enough primary sequence information to confer glucokinase catalytic activity to the chimera will be useful in this context. However, it will often be preferred to use the entire glucokinase enzyme as this is more straightforward in terms of methodology. Again, one may look to the extensive information available in various published references in order to assist with the identification of appropriate glucokinase enzymes or fragments thereof.

At this point, a discussion of the kinetic properties of hexokinase and glucokinase is relevant. It will be understood that in providing a functional equivalent of a hexokinase or glucokinase enzyme, one would desire to provide a protein that has substantially the same kinetic parameters as the native enzyme. Equally, in providing a hexokinase mutant

that is devoid of catalytic activity, one would provide an enzyme that is either completely inactivated or whose kinetic parameters have been shifted so that it is, in fact, distinct from the native enzyme.

5 Table 1, below, sets forth a comparison of glucokinase with hexokinases I-III. This information may be used in order to determine whether any particular variant is "equivalent", and also, to confirm that any inactive mutants have indeed been properly disabled.

10 TABLE 1
A Comparison of Glucokinase With Hexokinases

	GK	HK 1-111
Km glucose	5-12 mM	0.02-0.13 mM
Km ATP	0.5 mM	0.2-0.5 mM
Ki G-6-P	60 mM	0.2-0.9 mM
Molecular weight	52 kd	100 kd
Substrate preference		
Glucose	1	1
Mannose	0.8	1-1.2
2-Deoxyglucose	0.4	1-1.4
Fructose	0.2	1.1-1.3

The activity of glucose as a substrate is taken as 1. The other numbers are expressed in relation to the activity of glucose as a substrate.

15 **Trehalose-6-Phosphate Metabolism.** In Baker's yeast, glucose phosphorylation is also catalyzed by a family of hexokinases that are related in sequence and function to the mammalian hexokinase gene family. Yeast cells, however, contain other genes

involved in carbohydrate metabolism for which there are no mammalian counterparts. The trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase complex is an example of such an activity.

5 The trehalose-6-phosphate synthase/phosphatase complex catalyzes the formation of trehalose, a disaccharide of two glucose molecules (α -D-glucopyranosyl (1-1) α -D-glucopyranoside) by first forming trehalose-6-phosphate by condensation of two molecules of glucose-6-phosphate and then using its phosphatase activity to remove the phosphate groups to generate free trehalose (Bell *et al.*, 1992). Trehalose is thought to
10 represent a form of storage polysaccharide in yeast, bacteria and other lower organisms, but neither the trehalose-6-phosphate synthase enzyme complex nor its products trehalose-6-phosphate or free trehalose are known to be present in mammalian cells.

 Blazquez *et al.* have demonstrated that trehalose-6-phosphate can inhibit the
15 activity of hexokinases from a variety of different organisms, including rat brain, which expresses predominantly hexokinase I (Blasquez *et al.*, 1993). This has led to the suggestion that trehalose-6-phosphate may be an important regulator of glycolytic flux in yeast cells. Consistent with this notion, the yeast gene known as *cif-1* was originally cloned from yeast that are unable to grow in glucose (Blasquez *et al.*, 1993) and
20 subsequently shown to be identical to the smallest subunit (56 kD) of the trehalose phosphate synthase/trehalose-6-phosphate phosphatase complex (Bell *et al.*, 1992). Cells lacking in the CIF-1 gene product exhibit rapid depletion of ATP, presumably because they are unable to produce trehalose-6-phosphate that normally serves to moderate yeast hexokinase activity. It is believed that the 56 kDa CIF-1 gene product
25 encodes the trehalose phosphate synthase activity (Bell *et al.*, 1992).

 One of the three general methods described in this application for inhibiting low K_m hexokinase activity in mammalian cells is to express an enzyme, such as yeast trehalose-6-phosphate synthase, that will allow trehalose-6-phosphate to accumulate.

30 This will have two effects. First, the accumulated trehalose-6-phosphate will serve to

allosterically inhibit endogenous low K_m hexokinase activity. Second, where trehalose-phosphate synthase is used, this enzyme will divert glucose-6-phosphate into trehalose-6-phosphate at low, non-stimulatory glucose concentrations where low K_m hexokinases but not glucokinases are active, thereby "short-circuiting" metabolic signaling for insulin secretion, which is thought to require ATP produced via further glucose metabolism (Newgard and McGarry, 1995).

A currently preferred gene for use in these aspects is the *S. cerevisiae* gene encoding trehalose-6-phosphate synthase (TPS1). Genes from several other organisms encoding trehalose-6-phosphate synthase have been isolated and the amino acid sequences deduced. These include *E. coli* (Accession # X69160), *S. pombe* (# Z29971), *Mycobacterium laprae* (# U15187) and *Aspergillus niger* (# U07184). It is contemplated that any of the foregoing or other biological functional equivalents thereof may be used in the context to the present invention.

Hexokinase Inhibition at Nucleic Acid Level Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down regulation of low K_m hexokinases include sequences from the Group I self splicing introns including Tobacco Ringspot Virus (Prody *et al.*, 1986), Advocado Sunblotch Viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne Transient Streak Virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead hammerhead ribozyme based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992 and Chowrira *et al.*, 1993) and Hepatitis Delta virus based ribozymes (Perrotta and Been, 1990). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira *et al.*, 1994, and Thompson *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozyme, the cleavage site is a dinucleotide sequence on the target RNA is a uracil (U) followed by either an adenine, cytosine or uracil (A,C or U) (Perriman *et al.*, 1992 and Thompson *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for low K_m hexokinases targeted here are greater than 3500 bases long, with greater than 500 possible cleavage sites.

The large number of possible cleavage sites in the low K_m hexokinases coupled with the growing number of sequences with demonstrated catalytic RNA cleavage activity indicates that a large number of ribozymes that have the potential to downregulate the low K_m hexokinases are available. Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in hexokinase-targeted ribozymes is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

Combination of Inhibitory Methods. Any of the three general methods of HK inhibition described above (Mitochondrial HK displacement, trehalose-6-phosphate generation and anti-HK ribozymes) may be combined with one another and/or with other

engineering methods. It is particularly contemplated that these methods could be used in combination with glucokinase overproduction. Glucokinase overproduction alone is even thought to be a useful method of inhibiting hexokinase, as set forth below.

5 Low K_m hexokinases, including hexokinases I and II that are present at high levels in mammalian cell lines, are inhibited by glucose-6-phosphate. Thus, this invention also relates to methods for maintaining glucose-6-phosphate at high levels in cell lines. The preferred method for achieving consistently high levels of glucose-6-phosphate in cells is to overexpress glucokinase in such lines.

10 Expression of glucokinase is considered advantageous for two distinct reasons. First, as described in U.S. Patent 5,427,940 expression of glucokinase is part of an advantageous method for engineering of glucose-stimulated insulin secretion in cell lines. Glucokinase expression is herein shown to have the added benefit of maintaining
15 high levels of glucose-6-phosphate to keep low K_m hexokinases in an inhibited state. This advantage would become particularly relevant at glucose concentrations in the physiological range (4-9 mM), because glucokinase is active at these levels. Also, while glucokinase is a member of the hexokinase gene family, it is not itself inhibited by glucose-6-phosphate.

20 **Advantages of Hexokinase Inhibition in Mammalian Cells.** The various aspects of this invention focus specifically on reducing the levels of low K_m hexokinase activity in mammalian cells. A particular type of target cell is a neuroendocrine cell. There are at least two significant achievements accomplished by the hexokinase
25 inhibition of the present invention, as set forth below.

30 In addition to the regulation of insulin secretion by glucose, the hexokinase gene family may also be important in the regulation of cell growth and proliferation. As described above, increases in low K_m hexokinase activity usually correlate with the transformation of cells from a normal to cancerous phenotype. However, the

correlation has not been proven to exist as a cause and effect relationship. In addition, increases in mitotic activity are not universally linked to induction of low K_m hexokinases. The activity of these enzymes did not increase in preneoplastic mouse beta cell lines over-expressing simian virus 40 large T antigen (Tag) (Radvanyi *et al.*, 1993);
5 nor are they universally elevated in fully transformed mouse β cells (Efrat *et al.*, 1993).

The reduction of hexokinase activity in a cell line by any suitable method, including any of the novel methods disclosed herein, is contemplated to be of use in inhibiting cell growth.

10 A relationship between low K_m hexokinase activity and cellular growth rates has three important implications relative to the application of cell-based therapies. First, from the perspective of iterative genetic engineering, an untimely or unregulated decrease of hexokinase activity will potentially hinder the growth and selection of clones
15 possessing desired genotypes and traits. A cell line that over-expresses hexokinase I from a regulatable promoter may provide the optimal genetic background for engineering of gene targets. For example, a RIN cell line could be developed that transgenically expresses hexokinase under the control of the tetracycline (Tet)-resistance operon regulatory system (Gossen and Bujard, 1992). This expression system allows
20 powerful transcription of gene products and permits the ablation of gene expression in the presence of Tet and Tet derivatives. Efrat *et al.* (1995) have demonstrated the feasibility of using this expression system to regulate large Tag gene expression. The expression of Tag caused transformation and expansion of mouse beta cells. A decrease of Tag expression, by the *in vitro* or *in vivo* administration of Tet, led to an inhibition of
25 cellular proliferation.

A RIN or neuroendocrine cell line that expresses HKI from a repressible promoter could be further engineered to express high levels of human insulin, glucokinase, and GLUT-2. In addition, such a cell line would be an ideal host for the
30 ablation or down regulation of low K_m hexokinases. Such engineering could be pursued

without the hindering complication of slowed growth. Following a series of desired genetic manipulations, the growth of the cells and the glucose sensing ability could be modulated by down regulating hexokinase expression.

5 A second implication of low K_m hexokinase as a regulator of cellular growth concerns the use of engineered cells for *in vivo* therapies. It is envisioned that cell-based delivery will be conducted by maintenance of the cells *in vivo* in a perm-selective device. It is contemplated that cells with reduced levels of low K_m hexokinase activity will survive for longer periods of time in devices or capsules as a consequence of their
10 reduced growth rates.

 A third implication of low K_m hexokinases as regulators of cellular growth involves the creation of novel β -cell lines. The over-expression of HKI by introduction of exogenous DNA into a primary beta cell could be an essential ingredient of the
15 transformation process. NIH-3T3 cells, an immortalized cell line, showed increases in glycolysis and growth rates following transfection with low K_m hexokinase (Fanciulli *et al.*, 1994). In a preferred embodiment, hexokinase I would need to be under the control of a promoter that can be down regulated. Such transcriptional regulation would allow the subsequent modulation of growth and glucose sensing.

20 A second important reason for reducing hexokinase activity is that it will contribute to the development of engineered cells that exhibit glucose-regulatable protein secretion, the most important aspect of which is presently the physiologically regulated release of insulin. Insulin release from the β -cells of the islets of Langerhans in the
25 pancreas is prominently regulated by the circulating glucose concentration. Glucose stimulates insulin release over the physiological range of glucose concentrations (approximately 4-9 mM), with the amount of insulin secreted being proportional to the rate of glucose metabolism (Newgard and McGarry, 1995).

Glucose phosphorylation appears to play an important role in regulating glucose metabolism and insulin responsiveness (Meglasson and Matschinsky, 1986). Thus, while islet extracts contain approximately equal amounts of high K_m glucokinase and low K_m hexokinase activities (Meglasson and Matchinsky, 1986; Hughes *et al.*, 1992), the hexokinases appear to be inhibited in intact islets, presumably by glucose-6-phosphate, allowing the glucokinase activity to be predominant. Since glucokinase has a K_m for glucose (approximately 6-8 mM) that is within the physiological range, it is ideally suited for regulating glycolytic flux and insulin release in proportion to the extracellular glucose concentration.

The concept of a regulatory role for glucokinase, which has been developed over several years (Meglasson and Matschinsky, 1986; Matschinsky, 1990), is supported by recent genetic and molecular studies, in which reduced expression of glucokinase was shown to result in less robust glucose-stimulated insulin secretion (Froguel *et al.*, 1993; Efrat *et al.*, 1994). Islet β -cells are also equipped with a specialized glucose transporter, GLUT-2, which like glucokinase is the high K_m member of its gene family.

One of the present inventors has shown that GLUT-2 and glucokinase work in tandem as the "glucose sensing apparatus" of the β -cell (U.S. Patent 5,427,940; Newgard *et al.*, 1990). U.S. Patent 5,427,940, incorporated herein by reference, describes methods for conferring glucose sensing in neuroendocrine cells and cell lines by transfection of such cells with one or more genes selected from the insulin gene, the glucokinase gene and the GLUT-2 glucose transporter gene, so as to provide an engineered cell having all three of these genes.

The overexpression of low K_m hexokinases is known to exert a dominant effect on the glucose concentration threshold for insulin secretion. Overexpression of a low K_m hexokinase from yeast in islet β -cells of transgenic animals results in increased rates of low K_m glucose metabolism and enhanced insulin release at subphysiological glucose concentrations (Epstein *et al.*, 1992; Voss-McGowan *et al.*, 1994). Similar changes

were noted upon overexpression of hexokinase I in isolated rat islets (Becker *et al.*, 1994a) or in all-differentiated insulinoma cell line called MIN-6 (Ishihara *et al.*, 1994).

5 It has been shown that the neuroendocrine cell lines that are contemplated for use in engineering artificial β -cells generally have significantly higher low K_m hexokinase activity than normal islet β -cells (Hughes *et al.*, 1992; Efrat *et al.*, 1993; Hughes *et al.*, 1993; Ferber *et al.*, 1994; Knaack *et al.*, 1994), and that glucose metabolism in such cells is highly active at low glucose concentrations. As the glucokinase:hexokinase activity ratio is a critical determinant of the glucose response threshold in insulin
10 secreting neuroendocrine cells, and as an imbalance in favor of hexokinase can cause insulin secretion to occur at glucose concentrations that are below the physiological threshold, it is evident that the most preferred artificial β cells should be further engineered to reduce hexokinase activity. The application of the methods of the present invention to the development of improved insulin secreting cells thus represents a
15 significant advance.

Inhibition Levels As defined herein, the degree of inhibition of hexokinase that is preferred is that necessary to achieve a glucose responsive insulin secretion in the physiologic range of 1.0 to 20 mM glucose. It will be understood by those working in
20 this field that the absolute level of inhibition is difficult to predict. Measurements of hexokinase and glucokinase in freshly isolated islets as well as cell lines varies dramatically. Ratios of HK to GK can vary from 2.8 (Burch *et al.*, 1981) to 0.8 (Liang *et al.*, 1990) to 0.5 (Hosokawa *et al.*, 1995) in fresh islets all with "normal" glucose stimulated insulin secretion. Reports of incorporated herein by reference cell lines with
25 "normal" secretion shows an HK to GK ratio of 0.6 (Efrat *et al.*, 1993) in the range of the fresh islets. These discrepancies illustrate the difficulties in specifying absolute numbers of glucokinase and hexokinase activities, hence the preference for using glucose responsive insulin secretion ranges as a meaningful parameter in this characterizing the cells invention.

(v) Methods for Re-engineering Engineered Cells

In many situations, multiple rounds of iterative engineering will be undertaken in generating the final cell lines. The events that may be conducted as separate construction events include blocking expression of endogenous gene products by molecular methods (including targeting of both copies of the endogenous gene), introducing a heterologous gene, and further modification of the host cell to achieve high level expression. The particular difficulty in performing multiple steps like this is the need for distinct selectable markers. This is a limitation in that only a few selectable markers are available for use in mammalian cells and not all of these work sufficiently well for the purposes of this invention.

The present invention therefore contemplates the use of the *Cre/Lox* site-specific recombination system (Sauer, 1993, available through Gibco/BRL, Inc., Gaithersburg, Md.) to rescue specific genes out of a genome, most notably drug selection markers. It is claimed as a way of increasing the number of rounds of engineering. Briefly, the system involves the use of a bacterial nucleotide sequence known as a *LoxP* site, which is recognized by the bacterial *Cre* protein. The *Cre* protein catalyzes a site-specific recombination event. This event is bidirectional, *i.e.*, *Cre* will catalyze the insertion of sequences at a *LoxP* site or excise sequences that lie between two *LoxP* sites. Thus, if a construct containing a selectable marker also has *LoxP* sites flanking the selectable marker, introduction of the *Cre* protein, or a polynucleotide encoding the *Cre* protein, into the cell will catalyze the removal of the selectable marker. If successfully accomplished, this will make the selectable marker again available for use in further genetic engineering of the cell. This technology is explained in detail in U.S. Patent No. 4,959,317, which is hereby incorporated by reference in its entirety.

It also is contemplated that a series of different markers may be employed in some situations. These markers are discussed in greater detail, below.

(vi) Leptin - Engineering Leptin Expression in Cells

In another embodiment of the present invention, the engineered cells may express and overexpress the obesity-associated protein known as leptin. Leptin is a peptide hormone that controls body composition and is believed to do so, at least in part, via interaction with hypothalamic receptors that regulate food intake and body weight. The various isoforms of leptin receptor (Ob-R), including the long isoform (OB-Rb), are widely expressed in various tissues, suggesting that leptin may play an important role in actions on extraneural tissues as well.

Additional evidence that leptin has non-neural function comes from a report that extraordinary changes in body fat are seen in rats made chronically hyperleptinemic by treatment with an adenovirus vector expressing the leptin cDNA. Chen *et al.*, *Proc. Nat'l Acad. Sci. USA* 93:14795 (1996). In this report, rats lost all discernible body fat within 7 days of adenovirus infusion, while animals that were "pair-fed" at the same low rate of food intake as the hyperleptinemic animals retain more of their body fat. The magnitude and rapidity of the lipid depletion suggested the possibility of a direct "hormone-to-cell" action by leptin, in addition to effects cause through the sympathetic nervous system.

Chen *et al.* (1996) also examined the effects of leptin overexpression on plasma glucose, insulin, plasma triglycerides and free fatty acid levels. While glucose did not change, both plasma triglycerides and free fatty acids dropped by about 50% in adenoviral-leptin treated animals, when compared to controls (Ad- β -gal or saline). These studies now have been confirmed and extended with respect to phospholipids. No clear cut changes in phospholipid concentration was observed. However, using an *in vitro* system, it was established that reductions in triglyceride levels could be achieved in the absence of sympathetic nervous system effects. Studies performed to determine what pathways are involved in the triglyceride depletion indicated that leptin-induced triglyceride depletion involves a novel mechanisms by which triglyceride disappears through enhanced intracellular triglyceride metabolism, rather than through more traditional free fatty acid export pathways.

Insulin levels in adenovirus-leptin infected rats dropped even more dramatically than the fatty acids, being only about 1/3 of the amount seen in controls. As stated above, the glucose levels of these animals was normal, however. These findings are consistent with enhanced insulin sensitivity in treated animals. Pancreata were isolated from hyperleptinemic rats and examined for β -cell function and morphology. The most striking finding was the complete absence of insulin secretion in response to either glucose or arginine. The morphology appeared normal, and it was determined that insulin secretion could be reestablished following perfusion of pancreatic tissue in the presence of free fatty acids, thereby establishing an important role for these molecules in β -cell function. These studies also indicate that leptin-mediated reduction of elevated tissue lipid levels will improve β -cell function, reduce insulin resistance and help restore abnormal glucose homeostasis in obese individuals.

A further connection between diabetes and leptin comes from studies with genetically obese ZDF rats, which contain mutant OB-R genes. The islets of these animals become overloaded with fat at the time that hyperglycemia begins. Because maneuvers that reduce islet fat content prevent diabetes in ZDF rats, it has been proposed that the accumulation of triglycerides in islets plays a causal role in β -cell dysfunction. Thus, the predisposition to diabetes in homozygous ZDF rats may reflect the fact that their tissue have been completely "unleptinized" throughout their life and therefore have accumulated high levels of TG. In normal rats, this accumulation is prevented by the action of leptin. It is expected that any therapy that reduces triglycerides in islets and in the target tissues of insulin will improve β -cell function and reduce insulin resistance.

In hyperleptinemic rats, every tissue that was examined was lipopenic. Thus, it is speculated that normal non-adipocytes carry a minute quantity of triglyceride, perhaps to serve as a reserve source of fuel in adipocytes that are depleted of fat by starvation and become unable to meet the fuel needs of certain tissues. It is suspected that this triglyceride storage function is closely regulated by leptin. In the obese ZDF rats, this

regulatory control is absent, and these putative intracellular triglycerides reserves soar to levels of over 1000-times that of hyperleptinemic rats.

In light of these observations, the present application therefore encompasses various engineered cells which express leptin in amounts in excess of normal. The methods by which leptin genes may be manipulated and introduced are much the same as for other genes included herein, such as amylin. A preferred embodiment would involve the use of a viral vector to deliver a leptin-encoding gene, for example, an adenoviral vector. This approach may be exploited in at least two ways. First, in the engineering of cells to produce certain polypeptides *in vitro*, it may be desirable to express high levels of leptin in order to downregulate various cellular functions, including synthesis of certain proteins. Similarly, leptin overexpression may synergize with cellular functions, resulting in the increased expression of an endogenous or exogenous polypeptide of interest.

Second, it may be desirable to use a leptin-overexpressing cell, or a leptin expression construct, such as a leptin-expressing adenovirus, in an *in vivo* context. This includes various "combination" approaches to the treatment of disease states such as obesity, hyperlipidemia and diabetes. For example, leptin expressing cell lines may provide for prolonged expression of leptin *in vivo* and for high level expression. Preliminary results indicate that injection of recombinantly produced leptin is less efficacious at achieving weight loss and reduction of lipids. Induction of hyperleptinemia using cells lines or expression constructs also may find use in reducing fat content in livestock just prior to slaughter. Moreover, because leptin-induced weight loss may act through different mechanisms than those currently employed, it may be possible to avoid related side effects such as diet-induced ketosis, heart attack and other diet-related symptoms. These regimens may involve combinations of other engineered cells, cells engineered with leptin and at least one other gene or genetic construct (knock-out, antisense, ribozyme, *etc.*), combination gene therapy or combination with a drug. The methods of delivering such pharmaceutical preparations are described elsewhere in this document

B. Proteins

A variety of different proteins can be expressed according to the present invention. Proteins can be grouped generally into two categories - secreted and non-secreted - discussions of each are detailed below. There are some general properties of proteins that are worthy of discussion at this juncture.

First, it is contemplated that many proteins will not have a single sequence but, rather, will exist in many forms. These forms may represent allelic variation or, rather, mutant forms of a given protein. Second, it is contemplated that various proteins may be expressed advantageously as "fusion" proteins. Fusions are generated by linking together the coding regions for two proteins, or parts of two proteins. This generates a new, single coding region that gives rise to the fusion protein. Fusions may be useful in producing secreted forms of proteins that are not normally secreted or producing molecules that are immunologically tagged. Tagged proteins may be more easily purified or monitored using antibodies to the tag. A third variation contemplated by the present invention involves the expression of protein fragments. It may not be necessary to express an entire protein and, in some cases, it may be desirable to express a particular functional domain, for example, where the protein fragment remains functional but is more stable, or less antigenic, or both.

(i) Secreted Proteins

Expression of several proteins that are normally secreted can be engineered into neuroendocrine cells. The cDNA's encoding a number of useful human proteins are available. Examples would include soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, TPA, urokinase, hirudin, interferons, TNF, interleukins, hematopoietic growth factors, antibodies, albumin, leptin, transferrin and nerve growth factors.

Peptide Hormones. Peptide hormones claimed herein for engineering in neuroendocrine cells are grouped into three classes with specific examples given for each. These classes are defined by the complexity of their post-translational processing.

Class I is represented by Growth Hormone, Prolactin and Parathyroid hormone. A more extensive list of human peptides that are included in Class I is given in Table 2.

These require relatively limited proteolytic processing followed by storage and stimulated release from secretory granules. Class II is represented by Insulin and Glucagon. A more extensive list of human peptide hormones that are included in Class II are given in Table 3. Further proteolytic processing is required, with both endoproteases and carboxypeptidases processing of larger precursor molecules occurring in the secretory granules. Class III is represented by Amylin, Glucagon-like Peptide I and Calcitonin. Again, a more extensive list of Class III human peptide hormones is given in Table 4. In addition to the proteolytic processing found in the Class II peptides, amidation of the C-terminus is required for proper biological function.

Examples of engineering expression of all three of these classes of peptide hormones in a neuroendocrine cell are found in this patent.

TABLE 2 - Class I Human Peptide Hormones

	Growth Hormone
	Prolactin
	Placental Lactogen
5	Luteinizing Hormone
	Follicle-stimulating Hormone
	Chorionic Gonadotropin
	Thyroid-stimulating Hormone
	Leptin

10

**TABLE 3 - Human Peptide Hormones Processed by
Endoproteases from Larger Precursors**

15	Adrenocorticotropin (ACTH)
	Angiotensin I and II
	β -endorphin
	β -Melanocyte Stimulating Hormone (β -MSH)
	Cholecystokinin
20	Endothelin I
	Galanin
	Gastric Inhibitory Peptide (GIP)
	Glucagon
	Insulin
25	Lipotropins
	Neurophysins
	Somatostatin

TABLE 4 - Amidated Human Peptide Hormones

Calcium Metabolism Peptides:	
5	Calcitonin
	Calcitonin Gene related Peptide (CGRP)
	β -Calcitonin Gene Related Peptide
	Hypercalcemia of Malignancy Factor (1-40) (PTH-rP)
	Parathyroid Hormone-related protein (107-139) (PTH-rP)
10	Parathyroid Hormone-related protein (107-111) (PTH-rP)
Gastrointestinal Peptides:	
	Cholecystokinin (27-33) (CCK)
	Galanin Message Associated Peptide, Preprogalanin (65-105)
15	Gastrin I
	Gastrin Releasing Peptide
	Glucagon-like Peptide (GLP-1)
	Pancreastatin
	Pancreatic Peptide
20	Peptide YY
	PHM
	Secretin
	Vasoactive Intestinal Peptide (VIP)
25	Pituitary Peptides:
	Oxytocin
	Vasopressin (AVP)
	Vasotocin
30	Enkephalins:
	Enkephalinamide
	Metorphinamide (Adrenorphin)
35	Alpha Melanocyte Stimulating Hormone (alpha-MSH)
	Atrial Natriuretic Factor (5-28) (ANF)
	Amylin
	Amyloid P Component (SAP-1)
	Corticotropin Releasing Hormone (CRH)
	Growth Hormone Releasing Factor (GHRH)
40	Luteinizing Hormone-Releasing Hormone (LHRH)
	Neuropeptide Y
	Substance K (Neurokinin A)
	Substance P
	Thyrotropin Releasing Hormone (TRH)

(ii) Non-Secreted Proteins

Expression of non-secreted proteins can be engineered into neuroendocrine cells.

Two general classes of such proteins can be defined. The first are proteins that, once expressed in cells, stay associated with the cells in a variety of destinations. These destinations include the cytoplasm, nucleus, mitochondria, endoplasmic reticulum, golgi, membrane of secretory granules and plasma membrane. Non-secreted proteins are both soluble and membrane associated. The second class of proteins are ones that are normally associated with the cell, but have been modified such that they are now secreted by the cell. Modifications would include site-directed mutagenesis or expression of truncations of engineered proteins resulting in their secretion as well as creating novel fusion proteins that result in secretion of a normally non-secreted protein.

Cells engineered to produce such proteins could be used for either *in vitro* production of the protein or for *in vivo*, cell-based therapies. *In vitro* production would entail purification of the expressed protein from either the cell pellet for proteins remaining associated with the cell or from the conditioned media from cells secreting the engineered protein. *In vivo*, cell-based therapies would either be based on secretion of the engineered protein or beneficial effects of the cells expressing a non-secreted protein.

The cDNA's encoding a number of therapeutically useful human proteins are available. These include cell surface receptors, transporters and channels such as GLUT2, CFTR, leptin receptor, sulfonylurea receptor, β -cell inward rectifying channels, *etc.* Other proteins include protein processing enzymes such as PC2 and PC3, and PAM, transcription factors such as IPF1, and metabolic enzymes such as adenosine deaminase, phenylalanine hydroxylase, glucocerebrosidase.

Engineering mutated, truncated or fusion proteins into neuroendocrine cells also is contemplated. Examples of each type of engineering resulting in secretion of a protein are given (Ferber *et al.*, 1991; Mains *et al.*, 1995). Reviews on the use of such

proteins for studying the regulated secretion pathway are also cited (Burgess and Kelly, 1987; Chavez *et al.*, 1994).

C. Genetic Constructs

Also claimed in this patent are examples of DNA expression plasmids designed to optimize production of the heterologous proteins. These include a number of enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in neuroendocrine cells. Elements designed to optimize messenger RNA stability and translatability in neuroendocrine cells are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable neuroendocrine cell clones expressing the peptide hormones are also provided, as is an element that links expression of the drug selection markers to expression of the heterologous polypeptide.

(i) Vector Backbone

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. In certain embodiments, expression includes both transcription of a gene and translation of mRNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a gene of interest.

In preferred embodiments, the nucleic acid encoding a gene product is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a particular gene is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the

control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

5 In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the gene of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a gene of interest is contemplated as well, provided that the levels
10 of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the gene product following transfection can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can
15 permit inducible expression of the gene product. Tables 5 and 6 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

20 Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA
25 with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An
30 enhancer region as a whole must be able to stimulate transcription at a distance; this

need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 5 and Table 6). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 5

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β -Interferon
Interleukin-2
Interleukin-2 Receptor
Gibbon Ape Leukemia Virus
MHC Class II 5 or HLA-DR α
β -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase I
Metallothionein
Collagenase
Albumin Gene
α -Fetoprotein
α -Globin
β -Globin
c-fos
c-HA-ras
Insulin Neural Cell Adhesion Molecule (NCAM)
α 1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40 or CMV
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus

TABLE 6

Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β -Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H ₂ O ₂
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TFA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

5 In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first

10 viruses used as gene vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive

15 cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic

material but can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

(ii) Regulatory Elements

5 Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the human Growth Hormone and SV40 polyadenylation signals in that they
10 were convenient and known to function well in the target cells employed. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

15 (iii) Selectable Markers

In certain embodiments of the invention, the delivery of a nucleic acid in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion of a drug selection marker aids in
20 cloning and in the selection of transformants, for example, neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable
25 of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

(iv) Multigene Constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

D. Bioreactors and Large Scale Cultures

The ability to produce biologically active polypeptides is increasingly important to the pharmaceutical industry. Over the last decade, advances in biotechnology have led to the production of important proteins and factors from bacteria, yeast, insect cells and from mammalian cell culture. Mammalian cultures have advantages over cultures derived from the less advanced lifeforms in their ability to post-translationally process complex protein structures such as disulfide-dependent folding and glycosylation. Neuroendocrine cell types have added unique capacities of endoproteolytic cleaving, C-terminal amidation and regulated secretion. Indeed, mammalian cell culture is now the

preferred source of a number of important proteins for use in human and animal medicine, especially those which are relatively large, complex or glycosylated.

Development of mammalian cell culture for production of pharmaceuticals has been greatly aided by the development in molecular biology of techniques for design and construction of vector systems highly efficient in mammalian cell cultures, a battery of useful selection markers, gene amplification schemes and a more comprehensive understanding of the biochemical and cellular mechanisms involved in procuring the final biologically-active molecule from the introduced vector.

However, the traditional selection of cell types for expressing heterologous proteins has generally been limited to the more "common" cell types such as CHO cells, BHK cells, C127 cells and myeloma cells. In many cases, these cell types were selected because there was a great deal of preexisting literature on the cell type (*e.g.*, "cookbook" methods for transfection of the cells) or the cell was simply being carried in the laboratory at the time the effort was made to express a peptide product. Frequently, factors which affect the downstream (in this case, beyond the T-75 flask) side of manufacturing scale-up were not considered before selecting the cell line as the host for the expression system. Also, development of bioreactor systems capable of sustaining very high density cultures for prolonged periods of time have not lived up to the increasing demand for increased production at lower costs.

The present invention will take advantage of the biochemical and cellular capacities of secretory cells as well as of recently available bioreactor technology. Growing cells according to the present invention in a bioreactor allows for large scale production and secretion of complex, fully biologically-active polypeptides into the growth media. By designing a defined media with low contents of complex proteins and using a scheme of timed-stimulation of the secretion into the media for increased titer, the purification strategy can be greatly simplified, thus lowering production cost.

(i) Anchorage-dependent versus non-anchorage-dependent cultures.

Animal and human cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing freely in suspension throughout the bulk of the culture; or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. Large scale suspension culture based on microbial (bacterial and yeast) fermentation technology has clear advantages for the manufacturing of mammalian cell products. The processes are relatively simple to operate and straightforward to scale up.

Homogeneous conditions can be provided in the reactor which allows for precise monitoring and control of temperature, dissolved oxygen, and pH, and ensure that representative samples of the culture can be taken.

However, suspension cultured cells cannot always be used in the production of biologicals. Suspension cultures are still considered to have tumorigenic potential and thus their use as substrates for production put limits on the use of the resulting products in human and veterinary applications (Petricciani, 1985; Larsson, 1987). Viruses propagated in suspension cultures as opposed to anchorage-dependent cultures can sometimes cause rapid changes in viral markers, leading to reduced immunogenicity (Bahnmann, 1980). Finally, sometimes even recombinant cell lines can secrete considerably higher amounts of products when propagated as anchorage-dependent cultures as compared with the same cell line in suspension (Nilsson, 1987). For these reasons, different types of anchorage-dependent cells are used extensively in the production of different biological products.

The current invention includes cells which are anchorage-dependent of nature. RIN cells, *e.g.*, are strictly anchorage-dependent, and when grown in suspension, the cells will attach to each other and grow in clumps, eventually suffocating cells in the

inner core of each clump as they reach a size that leaves the core cells unsustainable by the culture conditions. Therefore, an efficient means of large-scale culture of anchorage-dependent cells is needed in order to effectively take advantage of these cells' capacity to secrete heterologous proteins.

(ii) Reactors and processes for suspension.

Large scale suspension culture of mammalian cultures in stirred tanks was undertaken. The instrumentation and controls for bioreactors adapted, along with the design of the fermentors, from related microbial applications. However, acknowledging the increased demand for contamination control in the slower growing mammalian cultures, improved aseptic designs were quickly implemented, improving dependability of these reactors. Instrumentation and controls are basically the same as found in other fermentors and include agitation, temperature, dissolved oxygen, and pH controls. More advanced probes and autoanalyzers for on-line and off-line measurements of turbidity (a function of particles present), capacitance (a function of viable cells present), glucose/lactate, carbonate/bicarbonate and carbon dioxide are available. Maximum cell densities obtainable in suspension cultures are relatively low at about $2-4 \times 10^6$ cells/ml of medium (which is less than 1 mg dry cell weight per ml), well below the numbers achieved in microbial fermentation.

Two suspension culture reactor designs are most widely used in the industry due to their simplicity and robustness of operation - the stirred reactor and the airlift reactor.

The stirred reactor design has successfully been used on a scale of 8000 liter capacity for the production of interferon (Phillips *et al.*, 1985; Mizrahi, 1983). Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easy, has good mass transfer of gasses and generates relatively low shear forces.

Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. However, continuous processes based on chemostat or perfusion principles are available.

A batch process is a closed system in which a typical growth profile is seen. A lag phase is followed by exponential, stationary and decline phases. In such a system, the environment is continuously changing as nutrients are depleted and metabolites accumulate. This makes analysis of factors influencing cell growth and productivity, and hence optimization of the process, a complex task. Productivity of a batch process may be increased by controlled feeding of key nutrients to prolong the growth cycle. Such a fed-batch process is still a closed system because cells, products and waste products are not removed.

In what is still a closed system, perfusion of fresh medium through the culture can be achieved by retaining the cells with a fine mesh spin filter and spinning to prevent clogging. Spin filter cultures can produce cell densities of approximately 5×10^7 cells/ml. A true open system and the simplest perfusion process is the chemostat in which there is an inflow of medium and an outflow of cells and products. Culture medium is fed to the reactor at a predetermined and constant rate which maintains the dilution rate of the culture at a value less than the maximum specific growth rate of the cells (to prevent washout of the cells mass from the reactor). Culture fluid containing

cells and cell products and byproducts is removed at the same rate. These perfused systems are not in commercial use for production from mammalian cell culture.

(iii) Non-perfused attachment systems.

5 Traditionally, anchorage-dependent cell cultures are propagated on the bottom of small glass or plastic vessels. The restricted surface-to-volume ratio offered by classical and traditional techniques, suitable for the laboratory scale, has created a bottleneck in the production of cells and cell products on a large scale. In an attempt to provide systems that offer large accessible surfaces for cell growth in small culture volume, a
10 number of techniques have been proposed: the roller bottle system, the stack plates propagator, the spiral film bottles, the hollow fiber system, the packed bed, the plate exchanger system, and the membrane tubing reel. Since these systems are non-homogeneous in their nature, and are sometimes based on multiple processes, they suffer from the following shortcomings - limited potential for scale-up, difficulties in
15 taking cell samples, limited potential for measuring and controlling the system and difficulty in maintaining homogeneous environmental conditions throughout the culture.

Despite these drawbacks, a commonly used process of these systems is the roller bottle. Being little more than a large, differently shaped T-flask, simplicity of the
20 system makes it very dependable and, hence, attractive. Fully automated robots are available that can handle thousands of roller bottles per day, thus eliminating the risk of contamination and inconsistency associated with the otherwise required intense human handling. With frequent media changes, roller bottle cultures can achieve cell densities of close to 0.5×10^6 cells/cm² (corresponding to 10^9 cells/bottle or 10^7 cells/ml of
25 culture media).

(iv) Cultures on microcarriers

In an effort to overcome the shortcomings of the traditional anchorage-dependent culture processes, van Wezel (1967) developed the concept of the microcarrier culturing systems. In this system, cells are propagated on the surface of small solid particles suspended in the growth medium by slow agitation. Cells attach to the microcarriers and grow gradually to confluency of the microcarrier surface. In fact, this large scale culture system upgrades the attachment dependent culture from a single disc process to a unit process in which both monolayer and suspension culture have been brought together. Thus, combining the necessary surface for a the cells grow with the advantages of the homogeneous suspension culture increases production.

The advantages of microcarrier cultures over most other anchorage-dependent, large-scale cultivation methods are several fold. First, microcarrier cultures offer a high surface-to-volume ratio (variable by changing the carrier concentration) which leads to high cell density yields and a potential for obtaining highly concentrated cell products. Cell yields are up to $1-2 \times 10^7$ cells/ml when cultures are propagated in a perfused reactor mode. Second, cells can be propagated in one unit process vessels instead of using many small low-productivity vessels (*i.e.*, flasks or dishes). This results in far better utilization and a considerable saving of culture medium. Moreover, propagation in a single reactor leads to reduction in need for facility space and in the number of handling steps required per cell, thus reducing labor cost and risk of contamination. Third, the well-mixed and homogeneous microcarrier suspension culture makes it possible to monitor and control environmental conditions (*e.g.*, pH, pO_2 , and concentration of medium components), thus leading to more reproducible cell propagation and product recovery. Fourth, it is possible to take a representative sample for microscopic observation, chemical testing, or enumeration. Fifth, since microcarriers settle out of suspension easily, use of a fed-batch process or harvesting of cells can be done relatively easily. Sixth, the mode of the anchorage-dependent culture propagation on the microcarriers makes it possible to use this system for other cellular manipulations, such as cell transfer without the use of proteolytic enzymes, cocultivation

of cells, transplantation into animals, and perfusion of the culture using decanters, columns, fluidized beds, or hollow fibers for microcarrier retainment. Seventh, microcarrier cultures are relatively easily scaled up using conventional equipment used for cultivation of microbial and animal cells in suspension.

5 (v) Microencapsulation of mammalian cells

One method which has shown to be particularly useful for culturing mammalian cells is microencapsulation. The mammalian cells are retained inside a semipermeable hydrogel membrane. A porous membrane is formed around the cells permitting the
10 exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting membrane is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim
15 (1982) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of polyamino acid that ionically bonds to the surface alginate. Finally the alginate is reliquefied by treating the droplet in a chelating agent to remove the calcium ions.
20 Other methods use cells in a calcium solution to be dropped into a alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Microencapsulated cells are easily propagated in stirred tank reactors and, with
25 beads sizes in the range of 150-1500 μm in diameter, are easily retained in a perfused reactor using a fine-meshed screen. The ratio of capsule volume to total media volume can kept from as dense as 1:2 to 1:10. With intracapsular cell densities of up to 10^8 , the effective cell density in the culture is $1-5 \times 10^7$.

The advantages of microencapsulation over other processes include the protection from the deleterious effects of shear stresses which occur from sparging and agitation, the ability to easily retain beads for the purpose of using perfused systems. scale up is relatively straightforward and the ability to use the beads for implantation.

(vi) Perfused attachment systems

Perfusion refers to continuous flow at a steady rate, through or over a population of cells (of a physiological nutrient solution). It implies the retention of the cells within the culture unit as opposed to continuous-flow culture which washes the cells out with the withdrawn media (*e.g.*, chemostat). The idea of perfusion has been known since the beginning of the century, and has been applied to keep small pieces of tissue viable for extended microscopic observation. The technique was initiated to mimic the cells milieu *in vivo* where cells are continuously supplied with blood, lymph, or other body fluids. Without perfusion, cells in culture go through alternating phases of being fed and starved, thus limiting full expression of their growth and metabolic potential. The current use of perfused culture is in response to the challenge of growing cells at high densities (*i.e.*, $0.1-5 \times 10^8$ cells/ml). In order to increase densities beyond $2-4 \times 10^6$ cells/ml (or 2×10^5 cells/cm²), the medium has to be constantly replaced with a fresh supply in order to make up for nutritional deficiencies and to remove toxic products. Perfusion allows for a far better control of the culture environment (pH, pO₂, nutrient levels, *etc.*) and is a means of significantly increasing the utilization of the surface area within a culture for cell attachment.

Microcarrier and microencapsulated cultures are readily adapted to perfused reactors but, as noted above, these culture methods lack the capacity to meet the demand for cell densities above 10^8 cells/ml. Such densities will provide for the advantage of high product titer in the medium (facilitating downstream processing), a smaller culture system (lowering facility needs), and a better medium utilization (yielding savings in serum and other expensive additives). Supporting cells at high density requires extremely efficient perfusion techniques to prevent the development of non-

homogeneity. This means the use of highly sophisticated procedures and apparatus and has, until recently, been confined to a relatively small scale.

(vii) CelliGen™ bioreactor system

5 The development of a perfused packed-bed reactor using a bed matrix of a non-woven fabric has provided a means for maintaining a perfusion culture at densities exceeding 10^8 cells/ml of the bed volume (CelliGen™, New Brunswick Scientific, Edison, NJ; Wang *et al.*, 1992; Wang *et al.*, 1993; Wang *et al.*, 1994). Briefly described, this reactor comprises an improved reactor for culturing of both anchorage-
10 and non-anchorage-dependent cells. The reactor is designed as a packed bed with a means to provide internal recirculation. Preferably, a fiber matrix carrier is placed in a basket within the reactor vessel. A top and bottom portion of the basket has holes, allowing the medium to flow through the basket. A specially designed impeller provides recirculation of the medium through the space occupied by the fiber matrix for assuring
15 a uniform supply of nutrient and the removal of wastes. This simultaneously assures that a negligible amount of the total cell mass is suspended in the medium. The combination of the basket and the recirculation also provides a bubble-free flow of oxygenated medium through the fiber matrix. The fiber matrix is a non-woven fabric having a "pore" diameter of from 10 μm to 100 μm , providing for a high internal
20 volume with pore volumes corresponding to 1 to 20 times the volumes of individual cells.

In comparison to other culturing systems, this approach offers several significant advantages. With a fiber matrix carrier, the cells are protected against mechanical stress
25 from agitation and foaming. The free medium flow through the basket provides the cells with optimum regulated levels of oxygen, pH, and nutrients. Products can be continuously removed from the culture and the harvested products are free of cells and produced in low-protein medium which facilitates subsequent purification steps. Also, the unique design of this reactor system offers an easier way to scale up the reactor.
30 Currently, sizes up to 30 liter are available. One hundred liter and 300 liter versions

are in development and theoretical calculations support up to a 1000 liter reactor. This technology is explained in detail in WO 94/17178 (August 4, 1994, Freedman *et al.*), which is hereby incorporated by reference in its entirety.

5 A number of culturing parameters, used in conjunction the CelliGen™ system, have been demonstrated to play a role in increased production. For example, the CelliGen™ Plus reactor system, including the use of non-woven polyester fiber matrix (preferably, Fibra-Cel™) and centrifugal lift impeller (preferably, Fibra-Cel™) are system components that give improved yields. Also, several media formulations have
10 been employed with improved performance. For example, use of serum free medium is preferred, as is the use of cholesterol rich lipid extract (0.01% to 0.10%, volume to volume), ascorbic acid (from between about 0.001 to 0.100 mM), glutamate (rather than 2 mM glutamine) at 2 to 20 mM, preferably 4 mM, alpha ketoglutarate (rather than 2 mM glutamine) at 2 to 20 mM, preferably 4 mM, and the absence of growth promoting
15 factors.

viii) CellCube™

20 The Cellcube (Corning-Costar) module provides a large styrenic surface area for the immobilization and growth of substrate attached cells. It is an integrally encapsulated sterile single-use device that has a series of parallel culture plates joined to create thin, sealed laminar flow spaces between adjacent plates. The Cellcube module has inlet and outlet ports that are diagonally opposite each other and help distribute the flow of media to the parallel plates. The medium is constantly recirculated from the module through an
25 oxygenator and back to the cube. The external oxygenator provides a bubble free stream of oxygenated medium and allows for the additional control of the pH of the medium. With concurrent addition of fresh medium, medium with secreted product and wastes can be harvested continuously, retaining the cell population in the cube.

During the first few days of growth the culture is generally satisfied by the media contained within the system after initial seeding. The amount of time between the initial seeding and the start of the media perfusion is dependent on the density of cells in the seeding inoculum and the cell growth rate. The measurement of nutrient concentration in the circulating media is a good indicator of the status of the culture. When establishing a procedure it may be necessary to monitor the nutrients composition at a variety of different perfusion rates to determine the most economical and productive operating parameters.

Cells within the system reach a higher density of solution (cells/ml) than in traditional culture systems. Many typically used basal media are designed to support $1-2 \times 10^6$ cells/ml/day. A typical CellCube™ run with an $21\,000\text{ cm}^2$ surface, contains approximately 1.2 liters of media within the module. The final cell density can exceeds 2.5×10^6 cell/cm² or 5×10^7 cells/ml in the culture vessel. At confluence, depending on the cell line used, media required can vary anywhere from 4-16 module volumes per day.

The advantage of the CellCube™ system is that it to a large extent replicates the conditions the cells experience in T flask culture. This allows for very linear scale up of any culture that is successfully grown in flask culture without severe loss in per-cell performance.

E. *In Vivo* Delivery and Treatment Protocols

It is proposed that engineered cells of the present invention may be introduced into animals with certain needs, such as animals with insulin-dependent diabetes. In the diabetic treatment aspects, ideally cells are engineered to achieve glucose dose responsiveness closely resembling that of islets. However, other cells will also achieve advantages in accordance with the invention. It should be pointed out that the experiments of Madsen and coworkers have shown that implantation of poorly differentiated rat insulinoma cells into animals results in a return to a more differentiated state, marked by enhanced insulin secretion in response to metabolic fuels (Madsen *et*

al., 1988). These studies suggest that exposure of engineered cell lines to the *in vivo* milieu may have some effects on their response(s) to secretagogues.

The preferred methods of administration involve the encapsulation of the engineered cells in a biocompatible coating. In this approach, the cells are entrapped in a capsular coating that protects the contents from immunological responses. One preferred encapsulation technique involves encapsulation with alginate-polylysine-alginate. Capsules made employing this technique generally have a diameter of approximately 1 mm and should contain several hundred cells.

Cells may thus be implanted using the alginate-polylysine encapsulation technique of O'Shea and Sun (1986), with modifications, as later described by Fritschy *et al.* (1991). The engineered cells are suspended in 1.3% sodium alginate and encapsulated by extrusion of drops of the cell/alginate suspension through a syringe into CaCl_2 . After several washing steps, the droplets are suspended in polylysine and rewashed. The alginate within the capsules is then reliquified by suspension in 1 mM EGTA and then rewashed with Krebs balanced salt buffer.

An alternative approach is to seed Amicon fibers with cells of the present invention. The cells become enmeshed in the fibers, which are semipermeable, and are thus protected in a manner similar to the micro encapsulates (Altman *et al.*, 1986). After successful encapsulation or fiber seeding, the cells may be implanted intraperitoneally, usually by injection into the peritoneal cavity through a large gauge needle (23 gauge).

A variety of other encapsulation technologies have been developed that are applicable to the practice of the present invention (see, *e.g.*, Lacy *et al.*, 1991; Sullivan *et al.*, 1991; WO 91/10470; WO 91/10425; WO 90/15637; WO 90/02580; U.S. Patent 5,011,472; U.S. Patent 4,892,538; and WO 89/01967; each of the foregoing being incorporated by reference).

Lacy *et. al.* (1991) encapsulated rat islets in hollow acrylic fibers and immobilized these in alginate hydrogel. Following intraperitoneal transplantation of the encapsulated islets into diabetic mice, normoglycemia was reportedly restored. Similar results were also obtained using subcutaneous implants that had an appropriately constructed outer surface on the fibers. It is therefore contemplated that engineered cells of the present invention may also be straightforwardly "transplanted" into a mammal by similar subcutaneous injection.

Sullivan *et. al.* (1991) reported the development of a biohybrid perfused "artificial pancreas", which encapsulates islet tissue in a selectively permeable membrane. In these studies, a tubular semi-permeable membrane was coiled inside a protective housing to provide a compartment for the islet cells. Each end of the membrane was then connected to an arterial polytetrafluoroethylene (PTFE) graft that extended beyond the housing and joined the device to the vascular system as an arteriovenous shunt. The implantation of such a device containing islet allografts into pancreatectomized dogs was reported to result in the control of fasting glucose levels in 6/10 animals. Grafts of this type encapsulating engineered cells could also be used in accordance with the present invention.

The company Cytotherapeutics has developed encapsulation technologies that are now commercially available that will likely be of use in the application of the present invention. A vascular device has also been developed by Biohybrid, of Shrewsbury, Mass., that may have application to the technology of the present invention.

Implantation employing such an encapsulation technique are preferred for a variety of reasons. For example, transplantation of islets into animal models of diabetes by this method has been shown to significantly increase the period of normal glycemic control, by prolonging xenograft survival compared to unencapsulated islets (O'Shea and Sun, 1986; Fritschy *et al.*, 1991). Also, encapsulation will prevent uncontrolled

proliferation of clonal cells. Capsules containing cells are implanted (approximately 1,000-10,000/animal) intraperitoneally and blood samples taken daily for monitoring of blood glucose and insulin.

5 An alternate approach to encapsulation is to simply inject glucose-sensing cells into the scapular region or peritoneal cavity of diabetic mice or rats, where these cells are reported to form tumors (Sato *et al.*, 1962). Implantation by this approach may circumvent problems with viability or function, at least for the short term, that may be encountered with the encapsulation strategy. This approach will allow testing of the
10 function of the cells in experimental animals but obviously is not applicable as a strategy for treating human diabetes.

 Engineering of primary cells isolated from patients is also contemplated as described by Dr. Richard Mulligan and colleagues using retroviral vectors for the
15 purposes of introducing foreign genes into bone marrow cells (see, *e.g.*, Cone *et al.*, 1984; Danos *et al.*, 1988). The cells of the bone marrow are derived from a common progenitor, known as pluripotent stem cells, which give rise to a variety of blood borne cells including erythrocytes, platelets, lymphocytes, macrophages, and granulocytes. Interestingly, some of these cells, particularly the macrophages, are capable of secreting
20 peptides such as tumor necrosis factor and interleukin 1 in response to specific stimuli. There is also evidence that these cells contain granules similar in structure to the secretory granules of β -cells, although there is no clear evidence that such granules are collected and stored inside macrophages as they are in β -cells (Stossel, 1987).

25 It may ultimately be possible to use the present invention in combination with that previously described by the one of the present inventors (U.S. Patent 5,427,940, incorporated herein by reference) in a manner described for clonal cells to engineer primary cells that perform glucose-stimulated insulin secretion. This approach would completely circumvent the need for encapsulation of cells, since the patient's own bone
30 marrow cells would be used for the engineering and then re-implanted. These cells

would then develop into their differentiated form (*i.e.*, the macrophage) and circulate in the blood where they would be able to sense changes in circulating glucose by secreting insulin.

5 Alternatively, it may be desirable to introduce genetic constructs to cells *in vivo*. There are a number of way in which nucleic acids may introduced into cells. Several methods are outlined below.

(i) Adenovirus

10 One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

15 The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the
20 adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked
25 only to mild disease such as acute respiratory disease in humans.

30 Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The

early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kB of DNA. Combined with the approximately 5.5 kB of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kB, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone

and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

5

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

10

Recently, Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

15

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Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is

30

the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention.

The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9 - 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*,

1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

5 (ii) Retroviruses

10 The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

20 In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

(iii) Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV)

(Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

5 With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*,
10 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was
15 cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

20 (iv) Non-viral vectors

In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. As described above, the preferred mechanism for
25 delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium
30 phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987;

Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that

DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

Another embodiment of the invention for transferring a naked DNA expression
5 construct into cells may involve particle bombardment. This method depends on the
ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to
pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several
devices for accelerating small particles have been developed. One such device relies on
a high voltage discharge to generate an electrical current, which in turn provides the
10 motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of
biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have
been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require
15 surgical exposure of the tissue or cells, to eliminate any intervening tissue between the
gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular
gene may be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be
20 entrapped in a liposome. Liposomes are vesicular structures characterized by a
phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes
have multiple lipid layers separated by aqueous medium. They form spontaneously
when phospholipids are suspended in an excess of aqueous solution. The lipid
components undergo self-rearrangement before the formation of closed structures and
25 entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat,
1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro*
has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-
30 mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and

hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

5 In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989).

10 In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

15 Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

20 Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 25 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialoganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid encoding a gene in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson *et al.*, U.S. Patent 5,399,346, and incorporated herein in its entirety, disclose *ex vivo* therapeutic methods.

(v) Pharmaceutical Compositions

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions - either gene delivery vectors or engineered cells - in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or

dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active ingredients as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent growth of microorganisms.

The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The vectors and cells of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection also may be prepared.

These preparations also may be emulsified. A typical composition for such purposes

comprises a 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate-buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters, such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components in the pharmaceutical are adjusted according to well-known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal. The term "unit dose" refers to a physically discrete unit suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to produce the desired response in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject, and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

F. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Hexokinase I Targeted Disruption

Methods

Construction of gene replacement vector. A 15 kB clone, containing a portion of the rat hexokinase I (HKI) gene encompassing exon 1, about 0.2 kB of intron 1 and about 14.8 kB of sequence upstream of exon 1, was employed. Sequence and maps of this clone aided in the mapping of the HKI gene and in the isolation of homologous isogenic sequences from RIN genomic DNA. The novel 1082 base sequence of the non-transcribed rat HKI genomic DNA as well as the first 170 bases of HKI transcribed DNA (Schwab and Wilson, 1989) is given as SEQ ID NO:13. A plasmid vector providing positive and negative selection, pPolIIshort-neobPA-HSV-tk, is derived from the pGEM3Zf(+) backbone and contains a neomycin phosphotransferase gene (positive selection) and two tandem copies of herpes simplex virus thymidine kinase gene (HSV-tk) that provide negative selection in the presence of ganciclovir (Ishibashi *et al.*, 1993). pPolIIshort-neobPA-HSV-tk was modified to create pAT9 by creating a unique *NotI* site 5' of the Neo cassette (FIG. 1). A 873 base pair fragment was amplified from RIN genomic DNA using oligos (TTTCCCCTCGAGCACCGCCCGGAACAGTACC, SEQ ID NO:16 and GTTGC GCCTCGAGCATGCTGACGGTGGGGG, SEQ ID NO:17) to provide a short arm of homology to the HKI gene. The sequence extends 5' from the first methionine of exon 1 and is flanked by engineered *XhoI* sites.

In addition, a 1121 base fragment was amplified from RIN genomic DNA using oligos (GTTGGACTCGAGAGTCACCTAAGGGCCTATG, SEQ ID NO:18 and GTTGCGCCTCGAGCATGCTGACGGTGGGGG, SEQ ID NO:17), providing a longer
5 short arm to serve as a positive control for screening for homologous recombinants by PCRTM. The 873 and 1121 base pair PCRTM fragments were restricted with *Xho*I and subcloned into pAT9 at a unique *Xho*I site which is flanked by the Neo cassette and the copies of HSV-tk (FIG. 1), generating pAT21 and pAT22, respectively.

10 Southern blot analysis in RIN 1046-38 genomic DNA with a probe within intron 1 revealed a 16 kB *Kpn*I fragment. This fragment was enriched by sucrose density ultracentrifugation, modified with adapters to create flanking *Not* I sites, and subcloned into lambda Dash II (Stratagene, La Jolla, CA). Recombinant phages containing the fragment were isolated by plaque screening. The 16 kB *Not*I fragment was cloned into the
15 unique *Not* I site of pAT22 to provide a long arm of homology to the HKI gene (FIG. 1), generating pAT23, the HKI replacement vector.

Cell culture, electroporation, and drug selection. Various cell lines derived from the rat insulinoma RIN 1046-38 line (Clark *et al.*, 1990) were grown in Medium 199 with
20 Earle's salts, containing 11 mM glucose and 5% fetal bovine serum. Exogenous DNA was introduced into the cells by electroporation. RIN cell lines were grown to 50% to 75% confluence, harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and resuspended in PBS for counting. For each electroporation, 1×10^7 cells were pelleted by centrifugation at 1000 rpm for 2 minutes and resuspended in 0.4 ml cold
25 Electroporation Buffer (137 mM NaCl, 6.1 mM glucose, 5 mM KCl, 0.7 mM Na₂HPO₄, 20 mM Hepes, pH 7.0). DNA was added to the cell suspension to achieve a final concentration of 30-50 micrograms per ml. DNA was electroporated into cells in a 2 mm cuvette at 170 volts and 510 microFaradies using an Electro Cell Manipulator 600 (BTX, Inc., San Diego, CA) Cells were plated in non-selective medium and cultured for 2 to 3
30 days. Medium containing G418 at a final concentration of 500 micrograms per ml was

used for 14 days to select for clones integrated with the neomycin resistance marker. Following positive selection in G418, ganciclovir (Syntex Inc., Palo Alto, CA) at a final concentration of 6 μ M was used to selectively kill clones expressing HSV-tk. Ganciclovir was applied for 3 days; cells were then maintained in medium containing G418.

5 PCRTM assay for targeted recombinants. Following positive selection in G418 and negative selection in ganciclovir, clones were grown until visible by the naked eye. Individual colonies were picked, dispersed in trypsin, and divided between duplicate cultures into 96-well plates. Following 10 to 15 days in culture, cells of one duplicate
10 were rinsed in PBS and lysed by incubation at 37°C for 8 to 12 hours in fifty microliters of Lysis Buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM MgCl₂, 5.0 mM 2-mercaptoethanol, 6.7 μ M EDTA, 1.7 μ M SDS, 50 μ g/ml proteinase K, pH 8.8), (Willnow and Herz, 1994). Five microliters of lysate were used as a template in a twenty-five
15 microliter polymerase chain reaction (PCRTM) in 16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM MgCl₂, 5.0 mM 2-mercaptoethanol, 6.7 μ M EDTA, 1 mM each dNTP, 80 μ g/ml BSA, 0.8 μ g/ml of each primer, and 2.5 units Taq DNA polymerase. The amplification program consisted of 92°C, 40 seconds, 57°C 40 seconds, 75°C, 1 minute (40 cycles) and a final extension for 5 minutes at 75°C. The oligonucleotides used to
20 amplify disrupted HKI included a primer in the 3' end of the Neo cassette (5'GATTGGGAAGACAATAGCAGGCATGC3' SEQ ID NO:19, primer 1, FIG. 1 Ishibashi *et al.*, 1993) and a primer in the HKI gene upstream of the putative recombination site (5'AGTCGCCTCTGCATGTCTGAGTTC3' SEQ ID NO:20, primer 3, FIG. 1). The plasmid pAT22, containing the longer short arm of homology, served as a positive control in this PCRTM reaction. A second control PCRTM reaction was also
25 included using primer 1 and a primer in the HK1 gene downstream of the recombination site (5'CTTGAGCTCTTACATGGTGTACAG3' SEQ ID NO:21, primer 2, FIG. 1). This control PCRTM reaction should detect both homologous and random integrants of the HK1 replacement vector. Recombinants detected in the first screen were confirmed in a second PCRTM reaction for which no positive control plasmid exists. The absence of such a
30 control negates the possibility of a false positive due to contamination. The primers in this

secondary screen were primer 1 and primer 4 (5'TCCCCAGGCGTGGGGTAGAAG3' SEQ ID NO:22), an oligonucleotide upstream of the recombination site in the HKI gene (FIG. 1). PCRTM products analyzed either by gel electrophoresis or a slot blot assay. For electrophoresis, reaction products were fractionated in 1% agarose gels in Tris-borate/EDTA buffer (9 mM Tris-borate, 0.2 mM EDTA). DNA was visualized by staining in ethidium bromide. For slot-blots, reaction products were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl, and transferred to a nylon membrane using a 96-well blot apparatus (Scheichler and Schuell, Keene, N.H.). DNA was cross-linked to the membrane and HKI amplified products were detected by hybridization with ³²P-labeled oligonucleotides complementary to HKI and internal to primers used in the amplification reaction. Positive clones were replated in 96-well dishes to obtain densities of one cell per well. These clones were allowed to grow and assayed by PCRTM with the primers described above. This cycle of dilution cloning was repeated until all clones of a plating were positive in the assay.

Genomic Southern analysis. RIN clones that were positive by PCRTM for a disrupted allele of HKI were assayed by genomic Southern. Genomic DNA was isolated using reagents and protocols of the QIAamp Blood Kit (catalog number 29104, Qiagen, Inc., Chatsworth CA) Five to ten micrograms of DNA were digested with enzymes as indicated and fractionated through 0.8% agarose gels using TEAN buffer (0.04 M Tris-HCl, 0.025 M sodium acetate, 0.018 M NaCl, 25 mM EDTA, pH 8.15). Electrophoresis was conducted for 12 to 16 hours at 25 to 35 volts with recirculation of the buffer. DNA was visualized by staining with ethidium bromide. DNA in the gel was denatured for 30 minutes in 0.5 N NaOH, 1.5 M NaCl. Following neutralization in 1 M Tris-HCl, pH 7.5, 1 M NaCl for 30 minutes, DNA was transferred to a nylon membrane (Hybond-N+, Amersham) in 10x SSC (1X: 0.15 M NaCl, 0.015 M sodium citrate) and cross-linked to the membrane by ultraviolet radiation (UV Stratalinker 2400, Stratagene, Inc.). Radiolabeled probes (³²P) for hybridization to and detection of genomic fragments were synthesized as directed using the rediprime Random Primer Labeling Kit (RPN 1633, Amersham Life Sciences). Membranes were prehybridized and hybridized in Rapid-hyb

Buffer (NIF939, Amersham Life Sciences). All incubations and washes were performed in a Micro-4 Hybridization Oven (Hybaid Limited). Membranes were exposed to X-OMAT, AR5 film (Kodak) to obtain autoradiographic signals.

Results:

Prior to construction of a gene replacement vector, a comparison was made of the copy number of HKI alleles in rat versus RIN genomic DNA. DNA was digested with *Xba*I, Southern blotted, and probed with a radiolabeled fragment from intron 1 of the HKI gene. Autoradiography revealed equivalent signals derived from the rat and RIN HKI gene fragments. Presumably, these signals correspond to diploidy of the HKI gene in both the rat and RIN genomes. This conclusion is supported by data that show RIN-derived cell lines to have maintained a diploid state in their chromosomes. Karyotype analysis of RIN 1046-38 showed a distribution of 35 to 40 chromosomes with the normal rat complement being 42 chromosomes.

The HKI replacement vector (FIG. 1) was transfected into RIN cells in three separate electroporations (EP): EP81, EP86, EP95. These electroporations differ from each other in their temporal distributions, the identity of the parental cell line, and the number of clones screened from each (Table 7). EP81 was derived from a low passage RIN 1046-38 cell line. Of the 500 colonies screened, none were positive for disruption of an HKI allele. RIN-52/17, a RIN 1046-38 derived clone, was the parental line in EP86. One positive clone was detected in a screen of about 970 colonies. RIN-52/9, a cell line engineered to express high levels of rat glucokinase with *pcb7/GK* was used as a parental line in EP95. About 3200 clones were screened by PCRTM for the presence of a disrupted HKI allele. None were positive.

Potentially, the loss of an HKI allele could result in a growth disadvantage and thereby lead to a lower frequency of detecting HKI gene replacement events. To negate a potential metabolic disadvantage conferred by loss of HKI activity, efforts were made to create parental cell lines that overexpress rat glucokinase. Such parental lines could

potentially serve two functions - first, to prevent metabolic stress should phosphorylation of glucose became rate-limiting in transformed cell lines with diminished HKI activity; and second, to restore a high K_m glucose-phosphorylating activity to the RIN lines to shift glucose-responsive insulin secretion towards a more physiological range. RIN-52/17, the parental cell line in EP86, had previously been electroporated with a plasmid conferring hygromycin resistance and containing a copy of the rat glucokinase (GK) cDNA. RIN-52/17 was hygromycin resistant and was thought to express moderate levels of glucokinase from the transgene. Subsequent data confirmed resistance to hygromycin, but disproved expression of GK from the transgene (Table 7). About 1000 individual clones were screened from EP86. From this screen one clone, 86/X4, was positive by PCRTM. Clone 86/X4 was initially identified by amplification with primer 1 and primer 3. The molecular weight of the amplified product was equal to that derived from the plasmid control. Confirmation of this clone as containing a disrupted HKI allele was obtained by amplification with primer 1 and primer 4. No plasmid control exists for this PCRTM reaction, therefore, the product is not the result of contamination.

TABLE 7

Electroporation (EP) of RIN Cell Lines with a HKI Replacement Vector

EP	Parental line	DrugR, Parental	Transgene	Clones screened	+ by PCR TM
81	RIN 1047-38	(-)	(-)	500	0
86	RIN 52-17	HygroR	(-)	970	1
95	RIN 52-9	HygroR	rat GK	3200	0

Targeted disruption of HKI was attempted in various RIN lines, in the absence of presence of high levels of expression of rat glucokinase (GK) from a transgene. Cells expressing the transgene were first selected for resistance to hygromycin (HygroR) and then assayed by Western blotting for expression of exogenous rat GK.

The original positive culture of 86/X4 was passaged several times prior to dilutional plating for assessing the purity of the clonal population. 197 individual colonies were cultured in 96-well plates, allowed to grow to 50-70% confluence, trypsinized, and split into duplicate cultures. Cells from one set of cultures were lysed and screened by PCRTM using primers 1 and 3 (FIG. 1) and then reaction products were analyzed by a slot assay. Two clones were confirmed as containing a disrupted allele of HKI. This result demonstrates two things. First, the original culture that was identified as 86/X4 was a polyclonal rather than a monoclonal population. Second, the clone containing the disrupted allele of HKI seems to have a growth disadvantage compared to other cells in the population. This latter possibility is supported by observations of the growth rates of the purified HKI replacement clone. The pure 86/X4 grows significantly slower (about one-half as fast) than clones randomly integrated with the replacement vector.

Additional data verifying the identity of clone 86/X4 were derived by analysis of genomic DNA by Southern blotting (FIG. 2). DNA was digested with *EcoRI* and *NotI*, blotted, and hybridized with a probe upstream of the recombination site (hatched rectangle, FIG. 1). DNA from RIN 1046-38 cells (lane 1) and from RIN-52/17 randomly integrated with pAT23 (lane 2) produce a predicted signal of about 5.5kB in the autoradiograph. This signal corresponds to a homozygous, wild-type HKI gene. Clone 86/X4 produces two autoradiographic signals in the genomic Southern (lane 3): a 5.5 kB signal corresponding to a wild-type allele and an additional signal (about 4.6 kB), indicative of a HKI allele that has homologously recombined with the replacement vector.

EXAMPLE 2

Insulin Knockout

Methods:

Construction of gene replacement vector. The rat insulin I gene (Genbank accession number J00747) provided a template from which to create primers for amplifying sequences from RIN genomic DNA. A 590 base pair fragment 3' of the rat insulin gene and corresponding to positions 4830 to 5420 was amplified by polymerase

chain reaction (PCR™), subcloned, and used as radiolabeled probe. RIN genomic Southern using this probe revealed a *Bgl*II fragment of about 12 kB that extends three prime from position 1176. This fragment was enriched by sucrose density ultracentrifugation and subcloned into *Bam*HI sites of lambda Dash II vector (Stratagene).

5 Recombinant phages containing the fragment were isolated by plaque screening. A portion of this fragment extending from an internal *Spe*I site to a *Not*I site provided by the lambda Dash vector was used to provide a long arm of homology to RIN DNA in the context of a replacement vector (FIG. 3). A short arm of homology to RIN DNA (five prime of the rat insulin I gene) was derived by amplification of a fragment corresponding to nucleotides 1822 to 2860. This fragment, flanked by *Xho*I sites, was cloned into the replacement vector (FIG. 3).

15 The plasmid backbone (pSL9), used for creating a rat insulin I (RINS-1) replacement vector, provided several features designed to enhance and complement disruption of the rat insulin I gene. First, positive selection for integration of exogenous DNA into the RIN genome was provided by the gene encoding neomycin phosphotransferase. The expression of this gene is linked to the expression of human insulin by an internal ribosome entry site (IRES). This allows disruption of the rat insulin gene to be coupled to expression of human insulin cDNA. Secondly, negative selection, to 20 allow enrichment of targeted over random integration events, was provided by the expression of the type 2 rat glucose transporter (GLUT-2). The presence of a functional GLUT-2 renders cells susceptible to streptozotocin (STZ) toxicity (Schnedl *et al.*, 1994). Thirdly, a unique *Pac*I site at the distal end of the long arm of homology was used to linearize the vector prior to electroporation into RIN cells (FIG. 3).

25 Cell culture, electroporation, and drug selection. Culture conditions are as described above except that following positive selection in G418, STZ (1 mM for 2.5 h) was used to selectively kill clones expressing a functional Glut-2 transporter.

PCR™ assay for targeted recombinants. Following positive selection in G418 and negative selection in STZ, clones were cultured for about 3-4 weeks. Cells in each well were dispersed in trypsin and divided between duplicate cultures into 96-well plates. Following 10 to 15 days in culture, cells of one duplicate were rinsed in PBS and lysed by incubation at 37°C for 8 to 12 hours in 50 µl of Lysis Buffer (16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM MgCl₂, 5.0 mM 2-mercaptoethanol, 6.7 µM EDTA, 1.7 µM SDS, 50 µg/ml proteinase K, pH 8.8) (Willnow and Herz, 1994). Five microliters of lysate were used as a template in a 25 µl PCR™ in 16.6 mM ammonium sulfate, 67 mM Tris-HCl, 6.7 mM MgCl₂, 5.0 mM 2-mercaptoethanol, 6.7 µM EDTA, 1 mM each dNTP, 80 µg/ml BSA, 0.8 µg/ml of each primer, and 2.5 units Taq DNA polymerase. The amplification program consisted of 40 cycles at 92°C, 40 seconds, 57°C 40 seconds, 75°C, 1 minute and a final extension for 5 minutes at 75°C, and was performed in a 96-well thermocycler (HB-96V, MJ Research, Inc., Watertown, MA). The oligonucleotides used to amplify disrupted RINS-1 included a primer in the 3'-end of the Neo cassette (5'-CAACCGGTGGGACATTTGAGTTGC-3' SEQ ID NO:23, primer 1, FIG. 3) and a primer in the RINS-1 gene upstream of the putative recombination site (5'-CCAAGTCATTATAGAATCATAGTC-3' SEQ ID NO:24, primer 2, FIG. 3). The plasmid pRD1 was created to serve as a positive control in the PCR™ reaction. The backbone of pSL9 was ligated to an insert encompassing all of the short arm of homology and extending an additional 200 base pairs 5'. PCR™ products were analyzed using a slot-blot apparatus (part number 27560, Schleicher and Schuell). Reaction products were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 1.0 M Tris-HCl, pH 7.5, 1.5 M NaCl, and transferred to a nylon membrane. DNA was cross-linked to the membrane and RINS-1 amplified products were detected by hybridization with ³²P-labeled oligonucleotides complementary to RINS-1 and internal to primers used in the amplification reaction. Positive clones were replated in 96-well dishes to obtain densities of one cell per well. These clones were allowed to grow and assayed by PCR™ with the primers described above. This cycle of dilution cloning was repeated until all clones of a plating were positive in the assay.

RIN clones that were positive by PCR™ for a disrupted allele of RINS-1 were assayed by genomic Southern. Genomic DNA was isolated using reagents and protocols of the QIAamp Blood Kit (catalog number 29104, Qiagen, Inc., Chatsworth, CA). Five to ten micrograms of DNA was digested with enzymes as indicated and fractionated through 0.8% agarose gels using a TEAN buffer (0.04 M Tris-HCl, 0.025 M sodium acetate, 0.018 M NaCl, 25 mM EDTA, pH 8.15). Electrophoresis was conducted for 12 to 16 hours at 25 to 35 volts with recirculation of the buffer from the positive to the negative electrode. DNA was visualized by staining with ethidium bromide. DNA in the gel was denatured for 30 minutes in 0.5 N NaOH, 1.5 M NaCl. Following neutralization in 1 M Tris-HCl, pH 7.5, 1 M NaCl for 30 minutes, DNA was transferred to a nylon membrane (Hybond-N+, Amersham, Chicago, IL) in 10X SSC (1X: 0.15 M NaCl, 0.015 M sodium citrate) and cross-linked to the membrane by ultraviolet radiation (UV Stratalinker 2400, Stratagene, Inc.). Radiolabeled probes (³²P) were synthesized as directed using the rediprime Random Primer Labeling Kit (RPN 1633, Amersham Life Sciences). Membranes were prehybridized and hybridized in Rapid-hyb Buffer (NIF939, Amersham Life Sciences). All incubations and washes were performed in a Micro-4 Hybridization Oven (Hybaid Limited). Membranes were exposed to X-OMAT, AR5 film (Kodak) to obtain autoradiographic signals.

EXAMPLE 3

Human Insulin Expression

Methods:

Expression plasmid construction, general design. Initial expression plasmids were based on pCB6 and pCB7 (Brewer, 1994). These plasmids utilize the strong promoter/enhancer of the human Cytomegalovirus (CMV) immediate-early regulatory sequence to express inserted genes of interest. Efficient polyadenylation of transcribed messenger RNA is directed by the human growth hormone polyadenylation sequence. pCB6 encodes the Neomycin resistance gene conferring resistance to the neomycin analog G418, while pCB7 encodes the hygromycin resistance gene. Both resistant markers are transcribed by the SV40 early promoter.

A second expression plasmid was constructed with many of the same elements as pCB6. The open reading frame of the neomycin resistance gene was amplified with the polymerase chain reaction from pCB6 (Brewer, 1994) using oligos
5 (CCGGATCCCATGATTGAACAAGAT, SEQ ID NO:25 and
CCAAGATCTCGCTCAGAAGAACTC, SEQ ID NO:26). The resulting 816 bp
amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site
of pCMV8, generating pCMV8/NEO/hGH PolyA. pCMV8 was derived from pCMV4
(Anderson *et al.*, 1989) following removal of the alpha mosaic virus 4 RNA translational
10 enhancer and replacing it with the 5' leader sequence of the adenovirus tri-partite leader
(+14 to +154 of major late transcript) fused to a hybrid intron composed of the adenovirus
major late transcript 5'-donor site and a 3'-splice site from a variable region
immunoglobulin gene on a 409 bp *Eco*RI/*Pst*I fragment (SEQ ID NO:14, Kaufman and
Sharp, 1982). Secondly, a portion of the gene encoding the 5'-transcribed leader of the
15 human Glucose Regulated Protein 78 (GRP78) was amplified using the polymerase chain
reaction from pThu6.5 (corresponding to bases 372 to 594, Ting and Lee, 1988) using
oligos (CCGGATCCAGGTCGACGCCGCCAA, SEQ ID NO:27 and
CGAGATCTTGCCAGCCAGTTGG, SEQ ID NO:28), generating SEQ ID NO:11. The
5'-leader of human GRP 78 has been shown to direct internal initiation of translation
20 allowing for construction of functional polycistronic genes in mammalian cells (Macejak
and Sarnow, 1991). The 235 bp amplified product (SEQ ID NO:11) was restricted with
*Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8/NEO/hGH PolyA
generating pCMV8/IRES/NEO/hGH PolyA (FIG. 4B). Unique restriction endonuclease
sites exist (5'-SalI/XbaI/BamHI-3') for subcloning fragments into this expression plasmid
25 between the CMV promoter/intron and the internal ribosome entry site/NEO elements.
cDNA's or other open reading frames cloned into these sites are transcribed from the CMV
promoter into a bicistronic message containing the cDNA as the upstream open reading
frame and neomycin resistance (NEO) as the downstream open reading frame. Both open
reading frames are translated efficiently, linking neomycin drug resistance and expression
30 of the upstream gene of interest.

A final expression plasmid was designed for expression of genes of interest. The 5' elements found in pCMV8 composed of the 5' leader sequence of the adenovirus tripartite leader (+14 to +154 of major late transcript) fused to a hybrid intron composed of the adenovirus major late transcript 5' donor site and a 3' splice site from a variable region immunoglobulin gene (SEQ ID NO:14, Kaufman and Sharp, 1982) was removed by endonuclease restriction by *Sna*B1 and *Bam*HI and ligated into *Sna*B1 and *Bgl*II restricted pCB6 (Brewer, 1994), generating pCB6/intron (FIG. 4A). *Sna*B1 cuts uniquely in both plasmids at identical positions in the CMV promoter sequence. pCB6/intron has several unique endonuclease restriction sites for subcloning fragments downstream of the intron sequence and upstream of the hGH PolyA sequence (5'-*Xba*I/*Kpn*I/*Mlu*I/*Cla*I/*Bsp*DI/*Xba*I/*Bam*HI-3'). The neomycin resistance gene is transcribed using the SV40 promoter from an independent transcriptional unit encoded on the plasmid (Brewer, 1994).

Human insulin expression plasmid. A human insulin cDNA contained on a 515 base *Eco*RI fragment (SEQ ID NO:1, Bell *et al.*, 1979) was ligated into the *Eco*RI site of pBluescript (Stratagene, Inc., La Jolla, CA), generating pBS/INS. pBS/INS was digested with *Hin*DIII, located 5' of the insulin open reading frame, and *Bam*HI, located 3' of the Insulin open reading frame. The resulting 542 base fragment was ligated into pCB6 that had been restricted with *Hin*DIII and *Bam*HI, generating pCB6/INS. pCB6/INS was digested with *Bgl*II and *Bam*HI and the resulting 549 base fragment containing the human insulin cDNA (SEQ ID NO:1) was ligated into the *Bam*HI site pCMV8/IRES/NEO/hGH PolyA generating pCMV8/INS/IRES/NEO. The CMV promoter drives transcription of a bicistronic messenger RNA with human insulin encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in G418. The same 542 base *Hin*DIII/*Bam*HI fragment was also ligated into *Hin*DIII/*Bam*HI digested pCB7 generating pCB7/INS. Stable transfectants from this plasmid are selected in hygromycin.

A third insulin expression plasmids was also constructed. pCB6/INS was digested with *Bgl*II and *Bam*HI and the resulting 549 base fragment containing the human insulin cDNA (SEQ ID NO:1) was ligated into the *Bam*HI site of pCMV8/IRES/PURO/hGH PolyA, generating pCMV8/INS/IRES/PURO. The CMV promoter drives transcription of a bicystronic messenger RNA with human insulin encoded in the upstream open reading frame and the puromycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in puromycin.

Alternative promoter/enhancers utilized in human insulin expression plasmids.

The rat insulin 1 promoter fragment was isolated from pAC/RIP (a derivative of pACCMV.pLpA in which the rat insulin 1 promoter was substituted for the CMV promoter, Becker *et al.*, 1994) as a *Kpn*I/*Hin*DIII fragment (SEQ ID NO:12) corresponding to bases -412 to +1 relative to the start site of transcription. This fragment was ligated into *Kpn*I/*Hin*DIII digested pBlueScript (Stratagene, Inc.), generating pBS/RIP. pBS/RIP was digested with *Kpn*I, treated with Klenow fragment to blunt the end, then digested with *Eco*RI, generating a 450 base pair fragment containing the rat insulin 1 promoter. This fragment was ligated into pCMV8/INS/IRES/NEO that had been previously digested with *Spe*I, treated with Klenow and then digested with *Eco*RI, generating pRIP8/INS/IRES/NEO.

The rat insulin 1 promoter fragment (441 base pair *Kpn*I/*Hin*DIII fragment, SEQ ID NO:12) was also ligated into both *Kpn*I and *Hin*DIII digested pCB6/INS and pCB7/INS generating pCB6/RIP.INS and pCB7/RIP.INS, respectively. The CMV promoter fragment of both of these plasmids was removed by digesting with *Spe*I and *Bgl*II (removing bases -585 to +1 of the CMV promoter), treating with Klenow fragment and ligating to close, generating pRIP6/INS and pRIP7/INS. Stable transformants of pRIP6/INS are selected in G418 while stable transformants of pRIP7/INS are selected in hygromycin.

The rat insulin 1 gene promoter fragment (RIP) was also modified in an attempt to strengthen its transcriptional activity. The principal modification involved the attachment of varying numbers of mutant Far-FLAT minienhancers (FFE minienhancer) (German, *et al.*, 1992) to different positions within an intact RIP or to a RIP truncated at -205 (-205RIP). FFE minienhancers were constructed by generating oligonucleotides corresponding to the region of RIP between -247 and -196 (top strand, 5'-GATCCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATCGACTG ACCCTAGGTCTAA-3' SEQ ID NO:29; bottom strand, 5'-GATCTTAGACCTAGGGTCAGTCGATTATTAACAAGGGGCCAGATGGCCTGATG AAGG-3', SEQ ID NO:30). The underlined sequences at the ends of the oligonucleotides are *Bam*HI and *Bg*II recognition sites. The oligonucleotides were annealed and ligated in the presence of restriction enzymes *Bam*HI and *Bg*II. Since *Bam*HI and *Bg*II produce compatible DNA ends but can no longer be digested by *Bam*HI or *Bg*II, the only multimers that escaped *Bam*HI and *Bg*II digestion were ligated head-to-tail. FFE minienhancer dimers, trimers, etc. were separated by polyacrylamide gel electrophoresis and blunt-end cloned into the transient transfection vector, pBS/RIP/hGH, at either a *Xho*I site immediately upstream of -415 of the intact RIP, into an *Avr*II site at -206 of an intact RIP, or into an *Apa*I site immediately upstream of -205RIP. The number and orientation of FFE minienhancer repeats were verified by DNA sequencing. The stable transfection vector, pFFE3/RIP8/INS/IRES/NEO containing three copies of FFE minienhancers (FFE3, SEQ ID NO:15), was generated by inserting a blunt-ended *Kpn*I/*Hind*III FFE3/RIP into pCMV8/INS/IRES/NEO in which the CMV promoter was removed with *Spe*I and *Sac*I. pFFE6/RIP8/INS/IRES/NEO was constructed by inserting an *Apa*I/blunt-ended *Hind*III FFE6/RIP fragment into pRIP8/hGH polyA in which RIP was removed by *Apa*I/*Eco*RV. A *Bg*II/*Stu*I INS/IRES/NEO fragment was then inserted into pFFE6/RIP8/hGH polyA to complete pFFE6/RIP8/INS/IRES/NEO.

The rat insulin 1 gene intron (RIPi) was obtained by polymerase chain reaction from rat genomic DNA using oligonucleotides CTCCCAAGCTTAAGTGACCAGCTACAA, SEQ ID NO:31 and

GGGCAACCTAGGTACTGGACCTTCTATC, SEQ ID NO:32. These oligos produced a 185 bp product containing the 119 base pair RIPi (Cordell *et al.*, 1979) and a HindIII site on the 5'-end and a BamHI site on the 3'-end. The PCR™ product was digested with HindIII and BamHI and ligated into pNoTA/T7, whereupon it was removed with XbaI blunt-ended with Klenow, treated with HindIII and inserted into EcoRV/HindIII digested pRIP8/INS/IRES/NEO to generate pRIP8/RIPi/INS/IRES/NEO. pFFE6/RIP8/RIPi/INS/IRES/NEO was constructed by replacing the 5' adenovirus-immunoglobulin hybrid intron/INS/IRES of pFFE6/RIP8/INS/IRES/NEO with RIPi/INS/IRES from pRIP8/RIPi/INS/IRES/NEO. p(RIE)3/-85RIP/RIPi/INS/IRES/NEO contained three copies of the rat insulin 1 gene enhancer (RIE) fused to RIP truncated at -85. This plasmid was constructed by replacing a BsgRI/HindIII RIP fragment from pRIP8/RIPi/INS/IRES/NEO with an ApaI/HindIII (RIE)3/-85RIP fragment. Both the BsgRI and ApaI restriction sites were blunt-ended by Klenow polymerase.

The 2,000 base pair Class III human insulin-linked polymorphic region (ILPR), a region demonstrated to enhance transcriptional activity of the human insulin promoter (Kennedy *et al.*, 1995), was obtained from the phage lambda clone I-H1-3 (Owerbach and Aagard, 1984). A PstI/NcoI fragment containing the ILPR was treated with Klenow and inserted into a blunt-ended XhoI site immediately upstream of RIP to make pILPR/RIP8/INS/IRES/NEO. Orientation of the 14 bp repeats in the ILPR with respect to RIP was determined by DNA sequencing.

The human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH) was isolated by the polymerase chain reaction from human genomic DNA using oligos (GGGTCTAGAGGACCTGTTCCCACCG, SEQ ID NO:33 and GCCGAATTCGAGGAGCAGAGAGCGAAGC, SEQ ID NO:34). These oligos generated a 1143 base product corresponding to bases -1121 to +22 of the published sequence (Ercolani *et al.*, 1988) with the introduction of a unique XbaI site at the 5' end and a unique EcoRI site at the 3' end. The PCR™ product was digested with PstI (located at position -735 relative to start site of transcription), treated with Klenow, and then

digested with *EcoRI*. The resulting 757 base fragment was ligated into pCMV8/INS/IRES/NEO that had been previously digested with *SpeI*, treated with Klenow and then digested with *EcoRI*, generating pGAPDH8/INS/IRES/NEO.

5 The Rous Sarcoma Virus Long Terminal Repeat (RSV) was isolated from pREP4 (Invitrogen, Inc., San Diego, CA). A 637 base pair *SalI/PvuII* fragment containing RSV was isolated, treated with Klenow to blunt the ends and ligated into pCMV8/INS/IRES/NEO that had been previously digested with *SpeI* and *EcoRI* and treated with Klenow, generating pRSV8/INS/IRES/NEO.

10 Celligen™ Bioreactor Inoculation and Culture. EP18/3E1 cells were grown, split, and maintained in RPMI-1640 medium with 2 mM glutamine (JRH Bioscience, Lenexa, KS) supplemented with 5% fetal calf serum (JRH) and 0.125 µg/ml G418 (Gibco BRL, Gaithersburg, MD) in T75 culture flasks as described previously. A large scale bioreactor
15 (Celligen Plus™, New Brunswick Scientific (NBS), Edison, NJ) with dissolved oxygen electrode, pH electrode (both Ingold), and 4-gas proportional-integral-derivative (P-I-D) controller is set up for perfusion culture with a packed bed of polyester discs (Fibra-Cel®, Sterilin, England) and a centrifugal lift impeller (Cell Lift™, NBS). The reactor has a working volume of 1.25 liters and a packed bed volume of 0.7 liters containing 70 grams
20 of polyester discs. Cells are trypsinized and seeded into the reactor containing the same media composition as the maintenance media at a density of approximately 10⁶ cells per ml of working volume. After transfer, the cells are allowed to seed onto the bed material for 8 h with a low impeller speed of 50 rpm and no media perfusion. After seeding, the impeller speed is brought up to 80 rpm and the culture is maintained with no perfusion for
25 approximately 75 hours. Media perfusion is started and the flow rate is brought from 0 working volumes per day (WV/d) to 4 WV/d over the course of the following 500 hours. The perfusion rate is thereafter maintained constant at 4 WV/d. The perfusion media is RPMI-1640 with 2 mM glutamine which is then supplemented with 2 g/l glucose (final concentration of 4 g/l), 0.10% fraction V bovine serum albumin, 10 µg/ml human apo-
30 transferrin, 50 µM each of ethanolamine and o-phosphorylethanolamine, and 0.10%

cholesterol rich lipids from adult bovine serum (Clark and Chick, 1990) (all Sigma Chemicals, St. Louis, MO). The perfusion media contains no fetal calf serum or other full sera. At approximately 600 hours of culture, the media was further enriched with glucose to a final concentration of 6 g/l. The impeller speed was increased to 100 rpm after 200 hours of culture, to 120 rpm after 300 hours, and to 150 rpm after 700 hours. The culture temperature is maintained at 37°C, the dissolved oxygen level at 80% (indexed relative to saturation of air in 37°C phosphate-buffered saline), and the pH at 7.4. Glucose levels in the reactor are maintained in the range of 1-3 g/l by adjusting the perfusion rate and the glucose concentration in the fresh perfusion media. Cultures have been maintained successfully for as long as 2000 hours in the bioreactor under similar conditions.

Media samples were collected once daily and quantitatively analyzed for insulin secreted into the media by ELISA as previously described. Selected samples were qualitatively analyzed for insulin processing by HPLC analysis as previously described. Ammonia and lactate levels are monitored in the daily samples and analyzed using an automated analyzer (IBI Biolyzer, Johnson & Johnson, New Brunswick, NJ).

At the end of the culture, the reactor is opened and a representative number of polyester discs are sampled for quantitation of DNA and insulin content.

CellCube™ Inoculation and Culture. EP11/3E9 were grown, split, and maintained in RPMI-1640 medium with 2 mM glutamine (JRH Bioscience, Lenexa, KS) supplemented with 5% fetal calf serum (JRH) and 0.125 µg/ml G418 (Gibco BRL, Gaithersburg, MD) in T150 culture flasks as described previously. A CellCube™ module (Corning Costar, Cambridge, MA) is rigged complete with oxygenator equipped with dissolved oxygen electrode and pH electrode. A "25 Stack" module has a working volume of 1.25 liters and an area available for cell growth of 21 000 cm². Cells are trypsinized and seeded into the module containing the same media composition as the maintenance media at a density of approx. 25×10^3 cells per cm². Each side of the cube is seeded separately, and the cells are allowed to seed onto the inside of the module for 4 hours with no

recirculation and no media perfusion. After seeding, the recirculation is set to 0.25 working volumes/min and the culture is maintained with no perfusion with fresh medium for approx. 24 hours. Media perfusion is started and the flow rate is brought from 0 working volumes per day (WV/d) to 8 WV/d over the course of the following 8 days, matching the extrapolated cell number with a medium delivery of 1 ml/10⁶ cells/day. The perfusion rate is thereafter maintained constant at 8 WV/d. The perfusion media is RPMI-1640 with 2 mM glutamine and supplemented with 2 g/l glucose (final concentration of 4 g/l). The culture temperature is maintained at 37°C, and the dissolved oxygen level at 80% (indexed relative to air at 37 °C), and the pH at 7.4. Media samples were collected once daily and quantitatively analyzed for insulin secreted into the media by ELISA as previously described. Ammonia and lactate levels are monitored in the daily samples and analyzed using an automated analyzer (IBI Biolyzer, Johnson & Johnson, New Brunswick, NJ).

Cyclically Stimulated Secretion in the Bioreactor. At a point during the culture when the oxygen controller output has stabilized around 60, the culture is cyclically stimulated with addition of a 10X concentrated secretion-stimulation cocktail once every 24 hours. The addition of the cocktail yields final medium concentrations of 10 mM each of leucine, argine, and glutamine, 100 µM IBMX, and 100 µM carbachol (all from Sigma). At the beginning of every cycle, approximately one-tenth of the working volume is replaced with the 10X cocktail while the perfusion of fresh media is left unchanged. At 4 l/d of perfusion, e.g., the remaining concentration of cocktail after 24 h is less than 2% relative to the initial concentration due to the continuous dilution by the perfusion. Six samples were taken every 30 minutes, then four samples every hour.

Stable transfection of cell lines. Cells were transfected by electroporation as described above for the Hexokinase 1 knockout electroporations.

Insulin message primer extension analysis. Total RNA from RIN cell lines grown *in vitro* was isolated using RNeasy B RNA Isolation Reagent (Qiagen/Biotex Laboratories

Int.). Total RNA from RIN cell lines grown *in vivo* as tumors was isolated using TriReagent (Molecular Research Center, Inc.). Ten µg total RNA was hybridized to a 5' digoxigenin-labeled oligo (GCCAGCAGGGGCAGGAGGCGCATCCACAGGGCCAT, SEQ ID NO:35, Genosys Biotechnologies, Inc.) in 0.25 M KCl at 68°C for 15 min. This oligo hybridizes to the first 35 bases of the endogenous rat insulin I as well as the human insulin open reading frames. Primer extension reactions were then carried out with 2.5 units AMV Reverse Transcriptase in the supplied buffer (Promega, Inc.) supplemented with 0.8 mM dNTP's (Pharmacia, Inc.) and 100 µg/ml Actinomycin D (Sigma Chemical Co.) at 42°C for one hour. Extension products were precipitated, resuspended in 40% water/60% Formamide, heated to 100°C for 5 min and run on a 8% acrylamide/7M urea/1X TBE denaturing gel. Electrophoresed products were transferred to Qiabrane Uncharged Nylon Membrane (Qiagen, Inc.) using a Transphor Unit, TE50X (Hoefer, Inc., San Francisco, CA). Digoxigenin-labeled products were detected using the Genius 7 Non radioactive Detection System (Boehringer Mannheim) followed by exposure to Xomat-AR auto radiography film (Kodak). Primer extension of endogenous rat insulin I message generates a 91 base product (Cordell *et al.*, 1979), the human insulin transgene expressed from pCB6 generates a 101 base product and the human insulin transgene expressed from pRIP7 generates a 68 base product. Primer extension of the human insulin transgene expressed from pCMV8/INS/IRES/NEO generates a primary signal of 280 bases with three other minor premature termination signals of approximately 190, 130 and 115 bases.

Northern analysis. Total RNA was isolated as described above for the primer extension protocol. Ten µg total RNA was resolved on methyl mercury/1.5% agarose gels as described (Bailey and Davidson, 1976). Gels were subsequently stained with ethidium bromide (1 µg/ml in 0.5 M ammonium carbonate) to visualize RNA for integrity and loading consistency. RNA was electro transferred to nylon membranes as described for the primer extension protocol. Membranes were hybridized with digoxigenin-labeled RNA probes using the Genius Non-Radioactive Nucleic Acid Labeling and Detection System for filter hybridizations as described (Boehringer Mannheim). Full-length digoxigenin-labeled antisense probes corresponding to human insulin, rat glyceraldehyde

3-phosphate dehydrogenase (GAPDH) (corresponding to bases 21 to 1162 of published sequence, Fort *et al.*, 1985) and the neomycin resistance gene (control template supplied in Genius 4 Kit) were made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) using either T7 or T3 Polymerase. Exposures of chemiluminescent detected membranes were performed using Xomat-AR autoradiography film (Kodak). In some cases, blots were hybridized with a ^{32}P -labeled cRNA probe for human insulin.

Stimulated insulin secretion assay. Four million RIN cells were seeded in 9 ml media in 25 cm² flasks (butyrate-treated cells were seeded at 10⁶ cells). Cells were then cultured with daily media changes for one week with or without 1 mM butyrate until cells reached 70-80% confluency. Prior to assay, cells were incubated 2 times for twenty minutes at 37°C in RPMI media lacking glucose and supplemented with 0.1% BSA and 20 mM HEPES, pH 7.2. The basal incubation of cells was for 1 h at 37°C in 4 ml RPMI containing 0 mM glucose, 0.1% BSA, 20 mM HEPES and 100 µM diazoxide (Sigma Chemical Co.). Basal secretion samples were collected and aliquotted for insulin immunoassays and HPLC analysis of insulin species. This was followed by the stimulated incubation of cells for 1 hour at 37°C in 4 ml RPMI with 5 mM glucose, 0.1% BSA, 20 mM HEPES, 10 mM each leucine, arginine and glutamine, 100 µM carbachol (Sigma Chemical Co.) and 100 µM IBMX. Stimulated secretion samples were then collected and aliquotted. Cells were returned to a basal incubation for 1 hour at 37°C in 4 ml RPMI containing 0 mM glucose, 0.1% BSA, 20 mM HEPES and 100 µM diazoxide.

Cells were then collected for determination of insulin content and cell number by addition of EDTA to the media to a final concentration of 2 mM and pipetting up and down to remove cells. Twenty percent of the cell suspension was taken for determination of DNA content. The remainder of the sample was centrifuged at 220 x g for 5 minutes to pellet the cells. The cell pellet was resuspended in 0.5 ml cold 0.1 M acetic acid/0.1% BSA and sonicated on ice (Setting 2, Sonic Dismembrator 50, Fisher Scientific, Pittsburgh, PA). The sonicate was aliquotted for insulin immunoassays and HPLC analysis of insulin species.

Determination of DNA content and cell number. RIN cells are pelleted and PBS removed. 0.5 ml of DNA extraction buffer (2.0 M NaCl, 2.0 mM EDTA, 40 mM Phosphate buffer, pH 7.4) is added to RIN samples and the RIN cells are sonicated, on ice, for 30 seconds at ~30% power (Fisher 50 watt sonicator). Four microliters of sonicate are then diluted into 1 ml fresh DNA assay dye solution (TNE - 10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4, containing 0.1 µg/ml Hoechst dye 33258 (Polysciences or Molecular Probes), with calf thymus DNA as a standard (Clontech Inc.). Samples are read using a DNA fluorimeter (Hoeffer Scientific Instruments, Model TKO100). 6 µg genomic DNA per 10⁶ cells was used for the conversion from DNA content to cell number values.

HPLC analysis of insulin processing intermediates. Acid/ethanol extracts of whole cells or conditioned media was prepared and analyzed by high performance liquid chromatography as described (Halban *et al.*, 1986, Sizonenko and Halban, 1991). Immunoreactive insulin (IRI) species were quantitated by radioimmunoassay as described (Halban *et al.*, 1986).

Tumor formation of transfected RIN cell lines in nude rats. Six to 8 week old athymic Fisher nude rats (Strain F344/Ncr-mu from National Cancer Institute, Fredrick, MD) were housed in a sterile isolation facility with free access to sterile standard laboratory chow and water. Three million cells were injected subcutaneously at two different locations in 100 µl PBS using a 1.0 cc U-100 insulin syringe (Bectin Dickenson). Tumors were excised once they were palpable, excess fat and associated tissue dissected away. Samples frozen prior to processing. Body weight and bleeds for blood glucose determination were taken prior to injecting cells and throughout the course of the experiment. Blood glucose was measured using an IBI Biolyzer (Kodak, Eastman Chemical Co.).

Results:

Rat insulinoma cells have been engineered to produce high levels of human insulin. The RIN cell line was derived from a radiation-induced tumor (Gazdar *et al.*, 1980). The insulin secretory characteristics of the parental cells used in these studies, RIN 1046-38, have been described and shown to exhibit abnormal sensitivity to glucose (Clark *et al.*, 1990 and Ferber *et al.*, 1994). These cells secrete insulin at glucose concentrations of 50 μ M, secreting 2-10 ng rat insulin/million cells/hour. This level of insulin is well below levels produced by primary rat or human islets (Rhodes and Halban, 1988 and Marchetti *et al.*, 1994) or other reported rodent insulinoma lines (Asafari *et al.*, 1992, Knaack *et al.*, 1994). RIN 1046-38 cells were stably transfected with an expression plasmid containing a human insulin cDNA driven by the human cytomegalovirus promoter (pCB6/INS). One clone, R5C.I-17, was selected based on high insulin secretion and further characterized. FIG. 4A. shows the total immunoreactive insulin content as measured by RIA of R5C.I-17 versus the parental RIN cell line. R5C.I-17 has a total insulin content of 450 ng per million cells, 3-fold above parental RIN.

Chronic culture of rat insulinoma cells in sodium butyrate has been shown to increase endogenous insulin message, content and secretion (Swarovsky *et al.*, 1994). To determine if similar increases would result from a human insulin transgene in rat insulinoma cells, R5C.I-17 cells were cultured for 7 days in 1.0 mM sodium butyrate. Cell growth was retarded but continued over the course of the week. Insulin content was determined at the end of the week and showed a 3-fold increase per cell above the untreated cells (FIG. 4A), consistent with data on the increase in content of endogenous insulin (Swarovsky *et al.*, 1994). This higher level of human insulin content suggests that the RIN 1046-38 cells are capable of synthesizing and storing more human insulin. Sodium butyrate treatment is also used to transiently induce insulin production in the large scale bioreactor.

To increase the level of production of human insulin, R5C.I-17 cells were stably transfected a second time with pRIP7/INS. This expression plasmid utilizes the rat insulin

l promoter driving expression of human insulin. EP11.3E9 was identified based on an increased insulin production above R5C.I-17 and characterized further. The insulin content of EP11.3E9 is 1400 ng per million cells, four times higher than its parent, R5C.I-17 or RIN (FIG. 4A).

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Human insulin, like endogenous rat insulin, is secreted via the regulated pathway in the engineered RIN cell lines. Insulin secretion values for a one hour pre-incubation in buffer alone (basal values) followed by a one hour incubation in a cocktail of IBMX, glucose and amino acids (stimulated levels) are shown in FIG. 4B. Low basal insulin secretion is seen from R5C.I-17 and EP11/3E9, even with the human insulin transgene constitutively expressed by the CMV promoter. A higher basal secretion is seen from the butyrate-treated R5C.I-17 cells. However, in all lines, insulin release was significantly increased following stimulation to levels of 150, 425 and 450 ng per million cells per hour from R5C.I-17, butyrate-treated R5C.I-17 and EP11/3E9, respectively. Stimulated insulin release per hour ranges from 25 to 35% of the intracellular stores for all four RIN lines, a value consistent with primary islet data (Curry, 1986 and Li *et al.*, 1994). R5C.I-17 has maintained its insulin output through more than 100 population doublings without drug selection (approximately one year in culture).

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Results from transgenic animals (Schnetzler *et al.*, 1993) and from cell lines (Halban and Wollheim, 1980) have supported the idea of a physiological set point for insulin production in β -cells. However, a threshold or upper limit on insulin production in the current engineered RIN cells has not been observed.

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Human proinsulin is efficiently processed to mature insulin by rat insulinoma cells.

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Intracellular insulin species were isolated from parental RIN, R5C.I-17 and EP11/3E9 cells by acid extraction. Separation by HPLC of the insulin species produced by these cells was done as described (Halban *et al.*, 1986, Sizonenko and Halban, 1991). The analysis indicates that human insulin produced by the rat insulinoma is efficiently processed to mature insulin with very low detectable levels of pro-insulin or other

processing intermediates (FIG. 5A, FIG. 5B, FIG. 5C). Processing of human insulin is slightly less efficient compared to the processing of rat insulin at these levels of production. While the percentage of intracellular rat proinsulin and intermediates is 3 to 4% of total rat insulin in all cell lines examined, the percentage of intracellular human proinsulin and intermediates is 8% in R5C.I-17 and approaches 18% in EP11/3E9. The ability of RIN cell lines to efficiently process proinsulin to mature insulin in these engineered lines demonstrates the maintenance of the high levels of expression of the endoproteases PC2 and PC3, known to be responsible for insulin processing (Vollenweider *et al.*, 1995). This is an important feature of the RIN cell lines being developed.

Expression of human insulin transgene is stable *in vivo*. RIN cells injected subcutaneously into a nude rat will form tumors. These tumors can then be excised and analyzed for gene expression. As has been seen previously, the majority of the tumor mass is RIN cell in origin with only small numbers of cells being host-derived in the form of endothelium, fibroblasts, *etc.* (Schnedl *et al.*, 1994). This allows for a convenient model for analysis of both endogenous and transgene expression in RIN cells *in vivo*. In the absence of any restraint of tumor growth, time points are restricted to one to two months because RIN cells secrete increasing amounts of rat, and in our studies human insulin, leading to hypoglycemia. Blood glucose levels were monitored throughout the course of the experiment.

One and a half million R5C.I-17 cells were injected subcutaneously at two sites per animal. The animals quickly become hypoglycemic in 8 to 10 days following this dose of cells (FIG. 6). Following the onset of hypoglycemia, animals are maintained on glucose in their drinking water as well as IP glucose injections prior to surgery. One animal demonstrated a blood glucose rebound following removal of both tumors. This rebound after two days of exogenous insulin-induced hypoglycemia is followed by the rapid removal of the exogenous insulin source.

Nine tumor masses in all were isolated from four animals between days 13 and 31. These tumors ranged in size from 40 to 200 mg in wet weight. RNA was isolated from tumors and expression of several gene products was analyzed and compared to uninjected cells maintained in tissue culture (*in vitro*). Primer extension analysis was used to compare the rat and human insulin signals in the same samples. The same primer hybridizes efficiently to both messages, but upon primer extension gives two different size products which can easily be resolved and quantitated. The results of this analysis are shown in FIG. 7. The same amount of starting RNA was used in each reaction from either cells maintained *in vitro* or from tumors, but contamination of non-RIN cells may cause the rat and human insulin signals to be underrepresented in the tumor samples. No attempt was made to correct for this. The signal for the human insulin transgene driven by the CMV promoter was very constant throughout the time points examined. No diminution of signal was apparent, suggesting the *in vivo* environment had no deleterious effects over the course of this experiment. Subsequent experiments have analyzed tumors at time points of 48 days (versus 31 days here) with no diminution in CMV driven/human insulin transgene expression.

To further test the stability of the CMV driven transgenes *in vivo*, engineered RIN cells were implanted into nude rats and transgene expression assessed with time. Several independent cell lines were implanted into multiple animals, expressing at least three different transgenes. The use of independent cell lines with different integration sites should give an unbiased answer to the issue of CMV promoter stability in RIN cells in this particular model. Longer time points of 48 days have been analyzed with no reduction in CMV driven expression. The *in vivo* model of implanting RIN cells into nude rats is limited by the uncontrolled growth of the RIN cells as a tumor. All the RIN lines used here make endogenous rat insulin, with some also making human insulin so that the animals quickly become hypoglycemic. Efforts were made to maintain the animals blood glucose by administering glucose in the drinking water or by i.p. injections, allowing analysis of tumors at longer time points. Alternatively, lower doses of cells can be injected initially (3×10^5 rather than 3×10^6) which is how a 48-day tumor was generated

and analyzed for maintenance of gene expression. Unfortunately, this lower dose of injected cells leads to a more sporadic tumor growth, making it harder to generate samples for analysis.

5 Surprisingly, endogenous insulin expression increased in all nine *in vivo* samples examined. This was unexpected since all nine tumors were excised following periods of extreme hypoglycemia, conditions known to down regulate pancreatic β -cell insulin message (Giddings *et al.*, 1982 and Brunstedt and Chan, 1982). Comparison of the ratio of rat to human insulin signals changed from 0.73 \pm 0.6 for several *in vitro* samples to
10 1.87 \pm 0.17 for the nine tumor samples. The 0.73 *in vitro* ratio correlates very well with the ratio of rat to human insulin (1 part rat to 1.5 parts human) observed for the R5C.I-17 cell line (see FIG. 5 for mass ratios). The increased rat to human message signal in tumors is paralleled by an increased rat to human insulin content in tumors subjected to acid extraction and HPLC separation of the insulin species.

15 A similar result was obtained following injection of the high human insulin producing cell line, EP11/3E9, into nude rats. Animals became hypoglycemic while maintaining body weight over the course of the experiment (FIG. 6). Nine tumors were isolated between days 15 and 28 following injection. Primer extension analysis of RNA
20 isolated from the tumors allows for separation of three insulin messages in the EP11/3E9 line. Extension of the endogenous rat insulin message and the human insulin message driven by the CMV promoter produced the identical pattern as seen in R5C.I-17, the parent cell line to EP11/3E9. A third extension product results from expression of the human insulin transgene message by the rat insulin 1 promoter. Primer extension analysis
25 on the tumor samples as well as the cell lines maintained *in vitro* show human insulin driven by the CMV promoter is stable throughout the course of the *in vivo* experiment. Again, endogenous rat insulin is upregulated in the *in vivo* environment, even in the face of hypoglycemia. The human insulin transgene driven by the rat insulin promoter is expressed throughout the course of the *in vivo* experiment.

In vivo potency of engineered RIN cell lines. When the engineered RIN lines are growing as tumors in the nude rats, their secreted insulin eventually impacts on the blood glucose levels of the healthy animals causing hypoglycemia (FIG. 6). While parental RIN cells have endogenous rat insulin outputs that eventually lead to hypoglycemia, RIN cells engineered to overexpress human insulin should induce hypoglycemia either faster with the same number of cells or require a smaller tumor mass. The inventors measured the tumor mass needed to induce the initial hypoglycemia in a nude rat as an indicator of *in vivo* potency of the engineered RIN cells.

Tumors were removed from the nude rats injected with either the R5C.I-17, EP11/3E9 or parental RIN 1046-38 at the first sign of hypoglycemia. The time between injection of a constant number of cells to hypoglycemia varied from 12 to 13 days for R5C.I-17 and EP11/3E9 (FIG. 6) to 28 days for the parental cells. All of the lines grow at the same rates *in vitro*. A plot of the tumor mass *versus* the *in vitro* stimulated insulin secretion values for these lines (FIG. 4B) is shown in FIG. 8. The higher the *in vitro* insulin output (both rat and human insulin), the smaller the tumor mass needed to cause hypoglycemia.

Endogenous GLUT-2 expression in RIN cells is lost *in vivo*. The expression of several other genes in the tumor samples was analyzed and compared to RIN cells maintained *in vitro*. The results of the analysis of both endogenous genes and introduced transgenes is shown in FIG. 9. RNA from two independent tumor samples from day 24 and day 25 were combined (*in vivo* sample) and compared to RNA from R5C.I-17 cells maintained in tissue culture (*in vitro* sample). Message levels of endogenous glucokinase, hexokinase I, amylin, GAPDH, sulfonylurea receptor and IPF1, as well as message levels of human insulin and the neomycin resistance transgenes, were relatively unchanged following 24 to 25 days of *in vivo* passage of R5C.I-17 cells. In contrast, the message level of endogenous GLUT-2 detected in R5C.I-17 cells maintained *in vitro* is completely absent in tumors at the 24/25 day time point. This result was duplicated on a separate analysis for confirmation (GLUT-2 a and b, FIG. 9). Analysis of individual tumors from

day 13 through day 31 demonstrated expression of GLUT-2 was already absent at the earliest time point analyzed and remained absent throughout the remainder of the experiment.

5 Loss of *in vivo* GLUT-2 expression could have serious consequences for the performance of cell lines designed for insulin delivery to animals or patients with diabetes.

10 Stable transfection of insulin producing cell lines with the GLUT-2 cDNA has been shown to confer glucose-stimulated insulin secretion (GSIS), while transfection of the same cells with a related transporter, GLUT-1, has no such effect (Hughes *et al.*, 1992, 1993; Ferber *et al.*, 1994; U.S. Patent 5,427,940). Furthermore, loss of GLUT-2 expression has been documented in a large number of rodent models of type II diabetes (NIDDM) in which β -cell failure involving loss of GSIS is a cause of hyperglycemia (Johnson *et al.*, 1990; Orci *et al.*, 1990; Thorens *et al.*, 1990; Unger, 1991). Endogenous GLUT-2 expression is apparently down-regulated or extinguished under diverse physiological conditions. In addition to the studies cited above, in which animals were hypoglycemic, implantation of normal islets from db/- mice into diabetic, hyperinsulinemic db/db mice or db/- mice rendered diabetic and hypoinsulinemic by injection of the β -cell cytotoxin, streptozotocin, resulted in loss of GLUT-2 expression in the transplanted islets (Thorens, 1992b). These results suggest that loss of GLUT-2 may also be responsible for impaired glucose responsive insulin release in human islets transplanted into patients with Type I or II diabetes (Scharp *et al.*, 1994). Reduced GLUT-2 suppression in the face of hyperglycemia is surprising in light of recent studies demonstrating that the GLUT-2 promoter is activated by glucose in hepatocytes or the insulinoma cell line INS-1 (Waeber *et al.*, 1994). Overall, these studies strongly imply that the GLUT-2 promoter is adversely effected by various metabolic perturbations *in vivo*, and that this promoter element is not appropriate for use in directing stable expression of heterologous genes in cell lines.

30 FIG. 10 illustrates that GLUT-2 expression can, in fact, be maintained in RIN cells implanted into nude rats for relatively prolonged periods of time if the gene is stably

transfected under the control of a viral promoter such as CMV. A RIN 1046-38 clone expressing high levels of rat GLUT-2 driven by the CMV promoter was generated using pCB7/GLUT-2 (clone EP49/206) as previously described (Ferber *et al.*, 1994). Animals injected with RIN EP49/206 form solid tumors and become hypoglycemic, much as reported for animals receiving cells containing only the endogenous GLUT-2 gene. Unlike the untransfected cells, however, GLUT-2 mRNA levels are maintained at a high, constant level over the two time points sampled, 16 and 34 days (FIG. 10A). These particular cells also were stably transfected with plasmids containing the human insulin and glucokinase cDNAs under control of CMV, and transcript levels for these other transgenes were maintained in a stable fashion, analogous to GLUT-2. These results indicate that cell lines transfected with multiple genes under control of a strong viral promoter like CMV are able to maintain stable expression of all transgenes for prolonged periods of time when transplanted into animals. These results are surprising and would not have been expected in light of previous studies from other groups who have reported that strong viral promoters such as CMV or RSV are often down-regulated in the *in vivo* environment (Palmer *et al.*, 1989 and 1991, Scharfmann *et al.*, 1991, Challita and Kohn, 1994).

Increased insulin production using expression plasmids containing an internal ribosome binding site. A new insulin expression plasmid was designed that links the expression of the drug selection marker to the expression of insulin. The plasmid, pCMV8/INS/IRES/NEO, utilizes the CMV promoter to drive a bicistronic message containing the human insulin open reading frame upstream of the neomycin resistance open reading frame. Placed between the two reading frames is a portion of the 5'-transcribed leader of the gene encoding human Glucose Regulated Protein 78 (GRP78; Ting and Lee, 1988). The 5'-leader of human GRP 78 has been shown to direct internal initiation of translation (Internal Ribosome Entry Site, IRES) allowing for construction of functional polycistronic genes in mammalian cells (Macejak and Sarnow, 1991). In order to generate Neo-resistant cells with this plasmid, the human insulin message must also be present, increasing the number of RIN clones that express human insulin protein. Since

internal initiation of translation by IRES elements is less efficient than normal 5' cap-dependent initiation (Macejak and Sarnow, 1991), cells must express high levels of the bicistronic transgene in order to survive drug selection. In this way, it should be possible to directly select with G418 clones expressing high levels of human insulin.

5

Twenty-nine independent G418 resistant clones from an electroporation of parental RIN 1046-38 cells with pCMV8/INS/IRES/NEO were screened for insulin content following acid extraction as described. The results are shown in FIG. 11 with the insulin content of R5C.I-17 (450 ng/million cells, FIG. 4A) used for comparison. Twenty-nine out of 29 clones expressed detectable levels of human insulin with at least 10 out of 29 of the clones (34%) expressing levels of human insulin more than 2-times that of R5C.I-17. RNA was isolated from the 5 highest insulin producing clones and human insulin message analyzed using primer extension. Starting inputs of 10 and 3 μ g of RNA from these 5 clones, as well as from a polyclone from this electroporation, were compared to 10 μ g of RNA from R5C.I-17. In the 5 monoclonal as well as the polyclone, high levels of human insulin message were detected at the expected size of 280 base pairs with three other minor premature termination signals of approximately 190, 130 and 115 bases. Even with 3 μ g of input RNA, the human insulin signal is still comparable to the signal from 10 μ g of RNA from R5C.I-17, a level of human insulin message in these clones in line with the higher levels of insulin protein.

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One clone, EP18/3E1 has been further characterized. The insulin content of EP18/3E1 is 1300 ng per million cells with a stimulated insulin secretion rate of 500 ng/million cells/hour. These levels of insulin are comparable to those achieved in EP11/3E9, our highest insulin producing clone to date (FIG. 4). However, in contrast to previous insulin producing clones, EP18/3E1 and other high insulin producing clones (FIG. 11) were generated from one round of electroporation using a single expression plasmid. The utility of the expression plasmid pCMV8/INS/IRES/NEO is in both the high numbers of positive clones and the higher insulin outputs of individual clones. Also, only one drug selection marker was used as opposed to two in generating EP11/3E9.

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Introduction of a second human insulin transgene into R5C.I-17 cells produced 11/3E9, a cell line with higher insulin production. Similarly, a second insulin construct was expressed in 18/3E1 cells to produce clones with increased insulin output. The construct consists of the human insulin gene linked to the puromycin resistance gene and the transcription of the bicistronic message produced is controlled by the CMV promoter. Colonies of cells that grew after selection in 2 µg/ml of puromycin were screened for increased insulin output. FIG. 12A demonstrates the expression of human insulin RNA of both bicistronic transgenes, and the increased insulin content for 5 selected clones. The cell line EP111/220 exhibited the highest cellular insulin content (FIG. 12B) and secreted the most insulin. The EP111/220 clone when incubated with the stimulation cocktail of mixed nutrients and secretagogues (as for FIG. 5B) secreted 0.99 µg insulin/10⁶ cells-hour. Currently, EP111/220 represents the highest documented insulin secretion of our cells engineered with human insulin.

The insulin content and secretory output of human islets may be estimated from reports in the literature. The average human pancreas contains about 0.9 g of islets (K. Saito *et al.*, 1978) which equals 9×10^8 cells (Finegood *et al.*, 1995), and the average human pancreas contains 200 U of insulin (with a 3-fold range; Wrenshall *et al.*, 1952). Thus, when *in situ*, the insulin content of the average human islets approximately 0.22 U/10⁶ cells, or 8 µg/10⁶ cells. Freshly isolated human islets are reported (Eizirik *et al.*, 1992 and 1994) to contain 8-10 µg/6 µg DNA (6 µg = 10⁶ cells). The same authors report that after one week of culture human islets contain 4-5 µg/6 µg DNA and with stimulation secrete 459 ng/6 µg DNA/h. Freshly isolated rat islets, for comparison, are reported to contain 4-8 µg insulin/6 µg DNA, and with stimulation secrete 0.2 to 2 µg insulin/6 µg DNA (Tokuyama *et al.*, 1995; Rhodes and Halban, 1988; Nielsen, 1985). The functionally-normal mouse β-cell lines secrete 400-800 ng insulin/h upon stimulation, and contain 3-10 µg insulin/10⁶ cells (Miyazaki *et al.*, 1990; Radvanyi *et al.*, 1993; Knaack *et al.*, 1994). The values presented for human islet insulin content and secretion are expected to represent the higher end of the range, because human islets are known to be less potent

than rodent islets, both *in vitro* (Smith *et al.*, 1991) and *in vivo* (Jansson *et al.*, 1995). The cell line EP111/220 has an insulin content that appears to be 60-75% of the value presented for cultured human islets, while insulin secretion appears to surpass that of cultured human islets.

5 The humanized β -cell lines generated in these studies exhibit a number of unique characteristics. First they express only one of the two rodent insulin genes (Fiedorek *et al.*, 1990 and the inventors' data), which will be advantageous in knockout development of complete insulin-humanized β -cell lines. Second, the present engineered lines have the
10 capability to increase insulin secretion 10- to 20-fold in response to stimuli. This characteristic is similar to that of β -cell lines derived from SV40-T antigen transgenic mice such as MIN6 (Miyazaki *et al.*, 1990), and β HC (Radvanyi *et al.*, 1993) cell lines as well as normal β -cells (Curry, 1986). Third, these cells maintain essentially normal processing of human proinsulin, even though the exogenous protein is in excess of
15 endogenous rat protein. Normal processing is not present in INS-1 (Neerman-Arbez *et al.*, 1993) and β TC cells (Nagamatsu and Steiner, 1992) two β -cell lines that have been examined for this property. Finally, the present cell lines demonstrate that iterative introduction of the insulin gene provides an approach whereby human insulin output can be stably achieved which (at minimum) matches that of cultured human islets.

20 Analysis of other promoter/enhancer elements for driving insulin expression.

Several other enhancer/promoters were compared to the CMV enhancer/promoter for their ability to direct transcription of the same bicistronic message (5'-intron/hINScDNA/IRES/NEO/hGH/3'-polyA) in stably transfected RIN38 cells. These
25 promoters include the rat insulin 1 gene promoter (RIP), modified RIP (FFE/RIP), RIP linked with the rat insulin 1 gene intron (RIP/RIPi) in place of the hybrid adenovirus/immunoglobulin 5'-intron, the Rous Sarcoma Virus Long Terminal Repeat (RSV), the human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH), and the mouse metallothionein promoter (MT). Expression plasmids were constructed by
30 removing the CMV promoter found in pCMV8/INS/IRES/NEO and replacing it with the

promoter to be tested. In this way, message levels and insulin outputs from the RIN clones constructed with the various promoters can be compared directly.

RIP activity is approximately 30- to 50-fold lower than that of the CMV promoter in transiently transfected RIN38 cells. However, in stably transfected RIN38 cells, RIP activity is much closer to the activity of the CMV promoter. The level of human insulin (hINS) mRNA derived from pRIP8/hINS/IRES/NEO is, on average, approximately only 3- to 5-fold lower than levels obtained from stable RIN38 lines containing pCMV8/hINS/IRES/NEO. The Northern blot depicted in FIG. 13 demonstrates this result as the level of hINS mRNA from two pRIP8/hINS/IRES/NEO RIN lines, 2.18 and 2.38, is only 3-fold lower than the level of hINS mRNA from the pCMV8/hINS/IRES/NEO RIN line, EP18/3E1. As stated earlier, the EP18/3E1 line has a very high insulin content, approximately equivalent to that of a normal human β -cell. Therefore, in addition to the CMV promoter, RIP offers another choice as a strong transcriptional activator.

RIP also was modified in an attempt to make it an even stronger transcriptional activator. The principal modification made to RIP was the attachment of Far-FLAT mini-enhancers (FF mini-enhancer). The FF mini-enhancer is located between -247 and -198 of RIP and contains several cis-acting regulatory elements crucial for RIP activity in β cells (Karlsson *et al.*, 1987; Karlsson *et al.*, 1989). The FF mini-enhancer region contains both the Far box (-239 to -230) and the FLAT element (-222 to -208) which further consists of two adjacent regulatory motifs, FLAT F and FLAT E. When isolated from the rat insulin I gene promoter and multimerized to yield 5 linked copies, the FF mini-enhancer is almost as active as an intact RIP in transiently transfected β -cells (German *et al.*, 1992). Three base changes in the FLAT E motif at positions -209, -211, and -213 can further increase the activity of the FF minienhancer (now called FFE minienhancer) approximately 3-fold in transiently transfected β -cells (German *et al.*, 1992). A transient transfection system with RIN38 cells was set up for initial screening of modified RIP promoter/enhancers. Results from the transient transfections utilizing a human growth hormone (hGH) reporter gene demonstrated that two modified RIP enhancer/promoters were 5-fold more active

than RIP. The two modified RIP enhancer/promoters consisted of an intact RIP (-415 to +1) to which either three or six copies of FFE minienhancers had been attached just upstream of -415 of RIP (the FFE sixmer is in the reverse orientation with respect to RIP). Coexpression of the RIP transcription factor, IPF-1, along with either pFFE3/RIP/hGH or pFFE6/RIP/hGH produced an 8-fold increase in activity over that of RIP alone.

To test whether or not the FFE-modified RIP enhancer/promoters would increase RIP activity in stably transfected RIN38 cells to the same extent as was demonstrated in transiently transfected RIN38 cells, FFE3/RIP was placed into the 5'-intron/hINScDNA/IRES/NEO/hGH/3'-polyA stable-transfection vector. A large number of RIN38 cell lines containing pFFE3/RIP8/INS/IRES/NEO were analyzed for FFE3/RIP activity. A number of clonal lines expressed higher human insulin mRNA than was observed for the best pRIP8/INS/IRES/NEO lines. Phosphoimager analysis of the Northern blot shown in FIG. 13 demonstrated that FFE3/RIP clones 4.17 and 4.32 produced approximately 2-fold more hINS than the highest-producing RIP lines 2.18 and 2.38. Therefore, these data demonstrate that RIP activity was enhanced in stable RIN lines by the addition of 3 FFE mini-enhancers, although not to the same extent as was shown in the transient transfection system. pFFE6/RIP8/INS/IRES/NEO is currently being introduced stably into RIN38 cells. Attempts to stably coexpress IPF-1 are also underway and are discussed below.

A second modification to RIP occurred by placing the rat insulin 1 gene intron (RIPi) immediately downstream of the transcriptional start site. It was previously noted that RIP activity was significantly increased in transgenic mice and, to a lesser extent, in cultured β -cells when combined with RIPi. A large number of stable RIN38 lines transfected with pRIP8/RIPi/INS/IRES/NEO were established and examined for hINS mRNA levels. As was observed for the FFE3 minienhancer, on average, the addition of RIPi to RIP yielded a modest but significant increase in hINS mRNA levels. The RIP/RIPi line, 2.65, expressed a level of hINS mRNA equivalent to the CMV promoter line, 18/3E1, and three times more hINS mRNA than the 2.18 and 2.38 RIP lines (FIG.

13). Since the addition of either RIPi or the FFE mini-enhancers enhances RIP activity, then combining both RIPi and FFE mini-enhancers with RIP could result in an additive increase of overall RIP strength. To test this idea, pFFE6/RIP8/RIPi/INS/IRES/NEO has been constructed and stably transfected into RIN38 cells. p(RIE)₃/
5 85RIP/RIPi/INS/IRES/NEO, a plasmid which contains both RIPi and three full-length rat insulin 1 gene enhancers instead of mini-enhancers, has also been constructed and transfected into RIN38 cells. FFE6/RIP/RIPi did act as a strong transcriptional activator but was only slightly stronger than either FFE6/RIP or RIP/RIPi alone. Interestingly, the three tandemly linked full-length RIP enhancers were very weak transcriptional activators
10 when p(RIE)₃/85RIP/RIPi/INS/IRES/NEO was stably integrated into RIN38 cells. This had not been the case in transiently transfected RIN38 cells in which the three linked RIP enhancers produced high-level expression of a linked reporter gene.

Another RIP derivative, pILPR/RIP8/INS/IRES/NEO has also been constructed in
15 an attempt to generate a more potent insulin promoter. The human Class III insulin-linked polymorphic region (ILPR) is composed of 139 tandemly-repeated 14 bp sequences and lies immediately upstream of the human insulin gene promoter/enhancer (Owerbach and Aagaard, 1984). It has recently been demonstrated that the presence of the Class III ILPR significantly increases the transcriptional activity of the human insulin promoter/enhancer
20 (Kennedy *et al.*, 1995). Likewise, fusing the Class III ILPR to RIP may also increase RIP activity. pILPR/RIP8/INS/NEO has been constructed and stably introduced into RIN38 cells. Analysis of polyclonal and monoclonal lines containing pILR/RIP8/INS/IRES/NEO demonstrate that the human Class III ILPR had no significant effect on RIP activity.

25 pRIP8(O₂)₇ is a modified RIP that has been altered by inserting seven copies of the operator site [(O₂)₇] from the *E. coli* tetracycline (*tet*)-resistance operon between the RIP enhancer and promoter at position -85. The tetracycline-resistance operon regulatory system (Gossen and Bujard, 1992) is a binary system in which a transactivator protein is also required. The transactivator is a combination of the tet repressor (tetR), which binds
30 very tightly to tet operator sites, fused to the transcriptional activation domain of virion

protein 16 (VP16) from herpes simplex virus. Both pRIP8(O2)7/RIPi/INS/IRES/NEO and an expression plasmid containing the tetR-VP16 transactivator will be stably transfected into RIN38 cells. Precedence for this type of scheme was recently demonstrated when the activity of the already potent CMV promoter was increased another 10-fold by inserting seven tet operator sites between the enhancer and promoter followed by cotransfection with the tetR-VP16 transactivator (Liang *et al.*, 1995).

The transcriptional activity of promoters other than CMV, RIP, and RIP derivatives also has been analyzed. Stable RIN38 lines were established which contained the promoter from the Rous Sarcoma Virus Long Terminal Repeat (RSV) driving the standard hINS/IRES/NEO stable transfection vector. In general, the RSV promoter produced hINS mRNA levels roughly equivalent to those produced by RIP. Therefore, the RSV promoter, like the CMV promoter, RIP, and RIP derivatives, acts as a strong transcriptional activator in RIN cells in culture. The human glyceraldehyde-3-phosphate dehydrogenase promoter (GAPDH) was also tested in stably transfected RIN38 cell lines and found to be a weak transcriptional activator. In most GAPDH promoter lines, hINS mRNA was either barely or not detectable by Northern blot analysis.

Promoter stability *in vivo*. As described earlier for the CMV and RIP promoters, the activity for some of the RIP derivatives, RSV, and GAPDH promoters was analyzed *in vivo* by subcutaneous injection of engineered RIN 1046-38 lines into athymic Fisher nude rats. *In vivo* activity of RIP was also reanalyzed, but this time without the presence of a CMV driven transgene as was the case for RIN line EP11/3E9. Time points were again restricted to one to two months as most of the animals developed hypoglycemia by two weeks after injection. The data from these experiments is summarized below.

In vivo RIP activity was examined for two independent RIN lines containing the pRIP8/INS/IRES/NEO transgene. Each line was injected into two individual nude rats. Animals containing either line became hypoglycemic between one to two weeks after injection. Tumors were excised at different intervals, homogenized, and analyzed for

hINS mRNA levels by Northern blotting. The amount of hINS mRNA remained constant out to the longest examined time points, 31 days for line 2.18 and 36 days for line 2.38. Therefore, RIP activity remained stable throughout the length of the experiment. The same results were obtained for the modified RIP promoter/enhancers, RIP/RIPi and FFE3/RIP. Both RIP/RIPi and FFE3/RIP produced constant levels of hINS mRNA out to the longest time point of 49 days.

The activity of the RSV promoter appears to be attenuated *in vivo*. Despite the formation of medium to large tumors, neither animal injected with the 3.4 line became hypoglycemic even after 36 days. Presumably, if analyzed at later time points, these animals would become hypoglycemic due to the endogenous expression of rat insulin from the engineered RIN lines. Both animals injected with the 3.34 line eventually did become hypoglycemic but it took much longer (20 to 30 days) than it did for the RIP and modified RIP lines (10 to 15 days). These data suggest that although the RSV enhancer/promoter is a strong transcriptional activator in cultured RIN cells, it may be unsuitable to direct the expression of a linked transgene in RIN cells in an *in vivo* situation. Further *in vivo* testing of RSV promoter activity utilizing a transgene other than the human insulin cDNA is presently underway.

GAPDH promoter activity remained stable *in vivo* out to the longest time point of 22 days. Both animals injected with the 4.5 line (the GAPDH line that produced the highest level of insulin mRNA) started to become hypoglycemic by 13 to 15 days. This result was somewhat surprising based on the relatively low abundance of hINS mRNA expressed in this line.

A concern with the use of the viral promoters is their long-term stability of expression *in vivo*. There are numerous reports concerning loss of transgene expression *in vivo*, either following introduction of genes *in vivo* with recombinant viruses or introduction of genes into cells *ex vivo* followed by implantation of the cells *in vivo* (Palmer *et al.*, 1989 and 1991, Scharfmann *et al.*, 1991, Challita and Kohn, 1994). This

second scenario is analogous to the proposed use of the cell lines being developed here for therapeutic use.

5 Interestingly, the RSV promoter driving transgenes in RIN clones appear to be attenuated *in vivo*. The mechanism for this attenuation is not clear. Evidence suggests that some of the problems with long term stability of expression of transgenes driven by viral promoters is due to immune recognition and ultimately rejection of the engineered cells (Dai *et al.*, 1995, Yang *et al.*, 1994). Immune recognition could be directed against the transgene product itself or against other antigens expressed following introduction of the
10 transgenes (*i.e.*, low level viral protein expression from recombinant viral transductions). However, in these studies using nude rats, there is no immune rejection of the implanted cells.

Cell growth, insulin content, and processing in a Celligen™ Bioreactor. The
15 oxygen gas controller output is monitored throughout the run. It is an indirect indication of the cells' oxygen consumption rate. It rises steadily from around -40 at 0 hours to around 60 at approximately 500 hours where it stabilizes for the rest of the run. The rate of increase of the controller output correlates with an expected growth rate of the culture, and maximum level of 60 is consistent with achieving a cell density of $1.1-2.3 \times 10^8$ cells per
20 ml of bed volume. The cell densities are confirmed at the end of the culture. With a surface-to-volume ratio of $120 \text{ cm}^2/\text{cm}^3$, the polyester disc bed yields a surface cell density comparable to that obtainable in two dimensional T flask culture. It is important to note that the growth and the sustained densities in the reactor are achieved using a serum free media. High density cultures have been maintained problem free for up to 2000 hours in
25 serum free medium. This observation is novel and very useful in the design of a bulk process for production of biological pharmaceuticals.

Cells harvested from the reactor at the end of culture by trypsinization, plated onto
T75 culture flasks, and assayed for insulin secretion performance after 24 hours of culture,
30 show no significant difference relative to sister cells maintained in T75 flask culture,

suggesting that the bioreactor milieu is not changing the cells' phenotype in any detectable fashion and that the cells quickly readapt to culture in tissue culture flasks.

HPLC separation of samples collected mid-run at around 550 hours of culture showed effective insulin processing. The ratio of mature human insulin to human proinsulin was 92:8. This efficient processing is obtained from a culture that has reached a steady state of oxygen uptake, indicating no overall growth, and that is sustained in a serum free medium.

The bioreactor data indicates that the steady state environment in the reactor allows for growth of up to approximately 2×10^8 cells per ml bed, while maintaining pathways crucial for complete processing and storage of insulin.

Cell growth and insulin production in a CellCube™. With a yield of approx. 50×10^9 cell from a 288 hour run, a surface cell density approx. twice that obtainable in T flask cultures was achieved. The concentration of secreted insulin in the medium rose steadily through the run. Peak levels of insulin measured in the perfused media was approx. 1200 ng/ml, corresponding to approx. 18 ng/ 10^6 cells/hour. The rise in insulin titer was paralleled by an increase in the per-cell performance. Compared to secretion of cultures in T flasks of approx. 23 ng/ 10^6 cells/hour, the peak CellCube™ secretion numbers in this run represent 75% efficiency in per-cell performance.

EXAMPLE 4

Glucagon Expression and Protein Processing in RIN Cells

Methods:

Rat glucagon cDNA isolation. Total rat pancreatic RNA was reverse transcribed into total cDNA using AMV Reverse Transcriptase as recommended by the supplier (Promega, Inc., Madison, WI). A rat glucagon cDNA corresponding to bases 10 to 904 of the published sequence (Heinrich *et al.*, 1984) was amplified with the polymerase chain reaction from the pancreatic cDNA using oligos (CCACCTGTCTACACCTCCTCTC,

SEQ ID NO: 36 and GTAATCCAGGTGTCGTGACTGC, SEQ ID NO:37). The resulting 895 base PCR™ product was ligated into pNoTA/T7 as recommended by supplier (5 Prime to 3 Prime, Inc., Boulder, CO), generating pNoTAT7/Glucagon.

5 Cell culture. RIN 1046-38 (Gazdar *et al.*, 1980, and Clark *et al.*, 1990), Rin 1027-B2 and Rin 1046-44 (Philippe *et al.*, 1987) were grown in Medium 199 with Earle's salts, containing 11 mM glucose and supplemented with 5% fetal calf serum, 100 milliunits/ml penicillin and 100 µg/ml streptomycin. AtT-20-derived cell lines were cultured as described (Hughes *et al.*, 1992). Cells were passaged once a week using 0.05% trypsin-
10 EDTA solution and kept under an atmosphere of 95% air and 5% CO₂ at 37°C.

Northern analysis. Northern analysis of glucagon transcripts in cell lines was done as described above for human insulin message using a digoxigenin-labeled antisense probe generated from pNoTAT7/Glucagon using T7 polymerase as recommended by supplier
15 (Boehringer Mannheim, Inc.).

Results:

Endogenous glucagon message is expressed in a subset of cell lines. Glucagon, a 29-amino acid peptide hormone involved in the regulation of glucose and fatty acid
20 metabolism (Unger and Orci, 1981), is proteolytically processed from preproglucagon, a large polypeptide precursor. Expression of the message for preproglucagon is found in a number of cell types, most notably alpha cells of the pancreas and L cells of the intestine. Preproglucagon posttranslational processing differs in these cell types, giving rise to predominantly glucagon from the alpha cells and Glucagon-like Peptides I and II (GLP-I
25 and II) from L cells (Mojsov *et al.*, 1986). The reason for this differential production in alpha cells and L cells is due to differential levels of expression of the endoproteases PC2 and PC3 (Rouille *et al.*, 1995). The expression of these endoproteases is known to vary in other cell types as well (Day *et al.*, 1992), giving rise to cell-specific posttranslational processing of POMC into distinct hormone peptides.
30

Rat insulinoma cells have been shown to express the glucagon message (Philippe *et al.*, 1987). A series of RIN cell derivatives all originating from the same original insulinoma (Gazdar *et al.*, 1980) were screened for expression of endogenous glucagon message. Northern analysis of various cell lines probed for glucagon message demonstrated that several cell lines do not express endogenous glucagon message including the RIN 1046-38 line used in this patent, AtT-20 cells (a rat pituitary derived cell line serving as a negative control) and an independent RIN line, RIN 1027-B2 (Philippe *et al.*, 1987). However, RIN 1046-44 cells, again independently derived from the same original insulinoma, does express the glucagon message (Philippe *et al.*, 1987). The majority of permanent clones the inventors have developed from the RIN 1046-38 parental line do not express the glucagon message. However, occasional clones of RIN 1046-38, such as EP53/114, engineered to overexpress rat glucokinase, now expresses significant levels of endogenous glucagon message. Expression of glucagon is not related to the glucokinase transgene expression (or any other specific transgene), as other clones overexpressing glucokinase do not express endogenous glucagon.

Expression of the endogenous glucagon message in RIN 1046-38 cells does suggest that it is possible to express a glucagon transgene in these cells. Construction of a glucagon expression plasmid by cloning the preproglucagon open reading frame of pNoTAT7/glucagon into pCMV8/TRES/NEO/hGHPolyA followed by transfection into RIN 1046-38 would result in high-level expression of the glucagon transgene message. Examples of this for several other genes including human insulin, human growth hormone and rat amylin are given herein.

Post-translational processing of preproglucagon into glucagon is dependent upon the specialized functions found in cells with a regulated secretory pathway. This is true in the endogenous cells that normally make glucagon (pancreatic alpha cells) and, as demonstrated in this patent, is true for RIN 1046-38 cells. Expression of preproglucagon transgenes in a variety of cell lines has demonstrated cell-specific differences in processing (Drucker *et al.*, 1986). RIN 1046-38 cells have the capacity to produce, process, store and

secrete human insulin as demonstrated in the above example. This includes high endogenous expression of PC2 and PC3, endoproteases involved in processing both insulin and glucagon.

5 RIN 1046-38 cells also should process preproglucagon into GLP-I and II. Final maturation of GLP-1 involves C-terminal amidation by peptidylglycine alpha-amidating monooxygenase (PAM), discussed in further detail below. Engineering RIN cells to predominantly produce glucagon or GLP-1 is possible by molecular engineering. Processing of preproglucagon to glucagon is predominantly by the action of PC2, while
10 processing to GLP-1 is predominantly by PC3. Overexpression of either a PC2 or PC3 transgene could result in predominant expression one peptide hormone over another. Alternatively, mutations can be induced in the glucagon transgene such that the dibasic amino acid residues recognized by PC2 and PC3 are altered such that only glucagon or GLP-1 is capable of being processed to the mature, biologically active polypeptide.

EXAMPLE 5

Human insulin disulfide mutant production

Methods:

Human insulin disulfide mutant expression plasmid. The human insulin open
20 reading frame was amplified with the polymerase chain reaction from a human insulin cDNA using oligos 1 and 2 (CCGGGGATCCTTCTGCCATGGCCC, SEQ ID NO:38 and GGGCTAGATCTAGTTGCTGTAGTTCTCCAGCTGGTAGAGGGAGCAGAT GCTAGTACTGCATTGTTCCAC, SEQ ID NO:39) generating a 358 base product (SEQ ID NO:3). Oligo 1 introduces a *Bam*HI site 7 bases upstream of the initiator methionine of
25 insulin. Oligo 2 introduces a *Bgl*II site just downstream of the insulin stop codon and introduces two point mutations into the insulin coding region. These mutations change cysteine at position 96 and cysteine at position 109 to serines (SEQ ID NO:4). Both of these amino acid substitutions are in the insulin A chain and disrupt the two disulfide bonds normally formed between the A and B chains. The mutated insulin protein should
30 be expressed, targeted to the regulated secretory pathway and proteolytically processed to

human insulin A, B and C chain. Upon stimulated secretion, the three peptide chains would be released by the cell without the normal disulfide bonds between the A and B chain. As a control, the wild-type human insulin open reading frame was amplified with the polymerase chain reaction from a human insulin cDNA using oligos 1 and 3 (CCGGGGATCCTTCTGCCATGGCCC, SEQ ID NO:38 and GGGCTAGATCTAGTTGCAGTAGTTCTC, SEQ ID NO:40). Again, Oligo 1 introduces a *Bam*HI site 7 bases upstream of the initiator methionine of insulin. Oligo 2 introduces a *Bgl*II site just downstream of the insulin stop codon without introducing any changes into the insulin coding sequence. The resulting 358 base pair PCR™ products were cloned directly into pNoTA/T7 (Prime PCR™ Cloner Cloning System, 5 Prime to 3 Prime, INC.) generating pNoTA/T7/mutINS and pNoTA/T7/wtINS. These plasmids were subsequently restricted with *Bam*HI and *Bgl*II endonucleases and ligated into *Bam*HI digested pCMV8/IRES/NEO/hGH PolyA, generating pCMV8/mutINS/IRES/NEO and pCMV8/wtINS/IRES/NEO, respectively.

A variation of pCMV8/mutINS/IRES/NEO was created by restoring the normal 3'-untranslated region of the insulin cDNA to its correct position following the insulin disulfide mutant open reading frame. An *Hga*I cleavage site is located 9 bases 3' of the insulin stop codon, base 364 of SEQ ID NO:1. pBS/INS was digested with *Hga*I, treated with Klenow fragment, and then digested with *Hind*III. The resulting 198 base pair fragment was ligated into pNoTA/T7/mutINS that had been digested with *Bgl*II, treated with Klenow fragment, and then digested with *Hind*III. The resulting plasmid, pNoTA/T7/mutINS+INS3', contains an essentially restored human insulin cDNA except for the two point mutations introduced into the coding region and a 5 base deletion at the *Bgl*II/*Hga*I cloning junction. This 198 base pair fragment contains 64 bases of the insulin 3'-untranslated region, a 41 base pair poly A tract, a 16 base pair poly C tract and 77 base pairs of polylinker sequence from the subcloning vectors. pNoTA/T7/mutINS+INS3' was digested with *Bam*HI, generating a 512 base fragment containing the mutant insulin and reconstructed insulin 3' sequence, which was ligated into the *Bam*HI site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/mutINS+3'/IRES/NEO.

Cell culture and stable transfection of cell lines. As described above for insulin producing cells.

5 Immunohistochemical staining for human insulin C-peptide. Individual G418-resistant RIN clones generated by electroporation using pCMV8/mutINS+3'/IRES/NEO were screened by immunostaining for human C-peptide. Cells were plated on multiwell slides one or more days before staining. Slides with spread cells were rinsed with PBS, then fixed 15-30 minutes in 4% paraformaldehyde. Fixation was followed by a PBS rinse
10 and permeabilization by passage through an ethanol series of 50%-70%-50% (5 minutes each). Permeabilization was followed by a PBS rinse and a 30 minute incubation in 50 mM Tris, pH 7.4, with 1% goat serum, 0.05% Triton and 0.1% azide. Slides were incubated with 1:10,000 dilution of rabbit anti-human C-peptide (Linco Inc.) for 24 hours.

Excess primary antibody was removed with sequential washes (3 minutes each) with
15 PBS-Triton (0.05%), PBS alone, and 50 mM Tris, pH 8.0. The slides were then incubated with an alkaline phosphatase-labeled second antibody (goat anti-rabbit IgG, Sigma Chemicals) in 50 mM Tris with 1% BSA and 1 mM magnesium chloride (Tris-BSA-Mg) for 30 minutes. Excess second antibody was removed with 3 washes of Tris-BSA-Mg. Alkaline phosphatase activity was then visualized by incubating 5 minutes in an alkaline
20 phosphatase substrate solution (BCIP/NBT).

Northern analysis. Northern analysis of mutant insulin transcripts in cell lines was performed as described above for human insulin message detection using a full-length digoxigenin-labeled antisense probe corresponding to the neomycin resistance gene
25 (control template supplied in Genius 4 Kit).

Results:

Recent reports suggest that immunomodulatory treatments with insulin can delay or prevent the onset of hyperglycemia in NOD mice (Shehadeh *et al.*, 1994; Sadelain *et al.*, 1990, and Muir *et al.*, 1993). Clinical trials evaluating the prophylactic nature of
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insulin in humans at high risk for the development of type I diabetes are underway (Keller *et al.*, 1993). Recently, immunization with metabolically inactive insulin B-chain also prevented the onset of hypoglycemia in NOD mice, suggesting an active induction of immunoregulation by insulin. Development of an *in vivo* cell-based delivery system of insulin or metabolically inactive forms of insulin could be used prophylactically in humans at high risk of developing type I diabetes. Cell lines producing and secreting high levels of mature human insulin have already been described here. This would be done in the context of the expression of reduced endogenous rat insulin. Neuroendocrine cells producing an inactive, mutant human insulin, in the context of reduced endogenous rat insulin production, would offer a safer, and possibly more efficacious approach. The use of metabolically inactive insulin would negate the possibility of insulin induced hypoglycemia. Higher amounts of a metabolically inactive insulin could therefore be safely administered *in vivo*, possibly increasing the efficacy of the treatment.

To this end, RIN cells have been engineered to produce a mutant form of human insulin. Insulin is initially produced in the cell as proinsulin, a larger peptide precursor consisting of the linear arrangement of insulin B-chain C-chain A-chain. The maturation of proinsulin to mature insulin is well understood (Halban, 1991) with three major steps in the process. The first is folding of the proinsulin into a native conformation in the immature secretory granules. The second step involves the formation of three disulfide bonds, one intramolecular in the A-chain and two intramolecular between the A-chain and the B-chain. The final step is the endoproteolytic processing by PC2 and PC3 followed by carboxypeptidase processing in the mature secretory granule. The mature granules contain an equimolar mix of C-chain (C-peptide) and mature insulin consisting of a A-chain/B-chain heterodimer covalently linked by the two intramolecular disulfide bonds. A mutant form of insulin was constructed from the human insulin cDNA in which the two codons encoding cysteins in the insulin A-chain have been mutated to codons encoding serines (SEQ ID NO:3). Expression of this mutant open reading frame should produce a mutant insulin peptide (SEQ ID NO:4) that still folds normally, the intrachain disulfide bond in the A-chain can still form, and endoproteolytic processing and carboxypeptidase cleavage

can still occur. The mature granules should now contain an equimolar mix of C-chain (C-peptide) and free B-chain and A-chain. The B-chain is identical in sequence to the wild-type human insulin B-chain used in studies showing the prevention of the onset of hypoglycemia in NOD mice (Muir *et al.*, 1995). Stimulated release of the contents of the secretory granules would release all three peptides. Engineering of these RIN cells in the context of reduced rat insulin production would ensure no insulin biologic activity.

EXAMPLE 6

Rat Amylin Production

Methods:

Rat Amylin expression plasmid. A *HindIII/XbaI* fragment corresponding to bases -66 to +611 of the published rat amylin cDNA sequence (SEQ ID NO:7, Leffert *et al.*, 1989) was treated with Klenow Fragment to blunt the ends. This blunt-ended fragment was ligated into the Klenow treated *XbaI* site of pCMV8/IRES/NEO/hGH PolyA generating pCMV8/Amylin/IRES/NEO. The CMV promoter drives transcription of a bicistronic messenger RNA with rat amylin encoded in the upstream open reading frame and the neomycin resistance gene encoded in the downstream open reading frame. Stable transfectants from this plasmid are selected in G418.

Cell culture and stable transfection of cell lines. RIN 1046-38 cells were cultured and transfected as described above for insulin producing cells.

Immunohistochemical staining for rat Amylin. As described above for human insulin C-peptide, with the following changes. The primary antibody was a rabbit anti-rat amylin polyclonal used at 1:1000 and 1:200 dilutions (Peninsula Labs, IHC 7323) for 80 minutes at room temperature.

Northern analysis. Northern analysis of rat amylin transcripts in cell lines was done as described above for human insulin message detection. Filters were hybridized with a full-length digoxigenin-labeled antisense probe corresponding to the rat amylin

cDNA (SEQ ID NO:7) made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) and T7 polymerase. Northern analysis of rat peptidylglycine alpha-amidating monooxygenase (PAM) in cell lines was done as described using a digoxigenin-labeled antisense probe corresponding to the bases 240 to 829 of the rat PAM cDNA (Stoffers *et al.*, 1989) made using Genius 4 RNA Labeling Kit (Boehringer Mannheim) and T7 polymerase.

Stimulated amylin secretion assay and determination of DNA content and cell number. This assay was performed as described for insulin secretion assay and cell number determination.

Amylin radioimmunoassays. Determination of rat amylin concentrations in stimulated and basal media samples was performed as previously described (Pieber *et al.*, 1994).

Results:

Peptidylglycine alpha-amidating monooxygenase expression in cell lines. Alpha-amidation is now appreciated as a critical determinant for biological activity of a large number of peptide hormones. Table 4 represents a sample of human peptide hormones that are known to be amidated *in vivo*. The enzyme involved in alpha-amidation, peptidylglycine alpha-amidating monooxygenase (PAM), has been well characterized at the molecular level (reviewed in Eiper *et al.*, 1992a). Although there is only one gene in mammals encoding PAM (Ouafik *et al.*, 1992), there are several forms of PAM due to alternative splicing and endoproteolytic processing (Stoffers *et al.*, 1989 and 1991, Eiper *et al.*, 1992b) leading to both membrane-bound and secreted forms of PAM. PAM is also known to be developmentally regulated and differentially expressed *in vivo* (Ouafik *et al.*, 1989). The importance of alpha-amidation of peptide hormones is such that the presence of the consensus glycine followed by two basic amino acids (lysine and/or arginine) in a novel amino acid sequence can be predictive of its being a precursor to a bioactive polypeptide (Cutitta, 1993).

Amylin and GLP-1 are two peptide hormones that are amidated *in vivo*. A more complete list of amidated human polypeptide hormones is found in Table 4. Attempts at mammalian cell production of any of these hormones requires endoproteolytic cleavage of larger precursors, carboxypeptidase trimming and alpha-amidation. For instance, Glucagon-Like Peptide 1 (GLP-1) is a peptide hormone with powerful insulintropic effects secreted from the intestinal L cells in response to meals (Kreymann *et al.*, 1987). It processed from a larger polypeptide precursor through steps that are very similar to the processing of amylin. Processing of GLP-1 involves the action of the endoproteases PC2 and PC3 and carboxypeptidase on the same precursor that glucagon (Mojsov *et al.*, 1986 and Rouille *et al.*, 1996). The final biologically active peptide is a mixture of GLP-1 7-37 and GLP-1 7-36 amide, a difference resulting from the alternative processing of the glycine at position 37 to an alpha-amidated form by peptidylglycine alpha-amidating monooxygenase (PAM) (Orskov *et al.*, 1989 and Mojsov *et al.*, 1990). Both GLP-1 7-37 and GLP-1 7-36 amide are both biologically active in humans (Orskov *et al.*, 1993). The rat insulinoma cell line used here, RIN 1046-38 has already been shown to express sufficient levels of PC2, PC3 and carboxypeptidase for complete processing of highly expressed human insulin.

Amylin is a 37 amino acid polypeptide hormone again processed from a larger precursor polypeptide by the proteolytic processing (Sanke *et al.*, 1988). Amylin is normally co-produced and co-secreted with insulin by b-cells, acting as a hormone to regulate carbohydrate metabolism (Hoppener *et al.*, 1994). However, unlike insulin, amylin is alpha-amidated by PAM in the b-cells (Sanke *et al.*, 1988). Overexpression of amylin in RIN 1046-38 cells will serve as a demonstration of the ability of these cells to produce amidated peptide hormones.

Northern analysis was used to address the endogenous levels of PAM in various cell lines. Expression of PAM in RIN 1046-38 is compared to AtT-20 and two related RIN lines, RIN 1027-B2 and RIN 1046-44 (Philippe *et al.*, 1987). Endogenous expression

of a single PAM message of approximately 3.5 kB is easily detected in all three RIN lines (FIG. 15A, Lanes 1, 3 and 4). Lower expression of two PAM messages of approximately 4.0 and 3.5 kB is found in AtT-20 cells (FIG. 15A, Lane 2). PAM message sizes of 3.5 to 4.0 kB is consistent with the larger spliced variants of PAM message known to encode active PAM protein (Stoffers *et al.*, 1989). Expression of endogenous PAM was compared with expression of endogenous amylin in these same cell lines. The three RIN lines with high levels of PAM also showed high levels of endogenous amylin expression (FIG. 15A, Lanes 1, 3 and 4). AtT20 cells, a pituitary cell line does not have any endogenous amylin expression. Interestingly, two RIN 1046-38 derived clones (EP18/3G8 expressing large amounts of human insulin (FIG. 11) and EP53/114 overexpressing rat glucokinase) that no longer express endogenous amylin show lower levels of expression of endogenous PAM (FIG. 15, Lanes 5 and 6). The majority of RIN 1046-38 derived clones continue to express both endogenous amylin and PAM, suggesting that RIN 1046-38 derived clones will maintain the ability to efficiently amidate peptide hormones.

The high level of PAM expression in RIN 1046-38 compared to AtT-20 is very encouraging. Comparison of PAM expression in other cell types has shown that AtT-20 cells express very high enzyme levels (Takeuchi *et al.*, 1990). This includes higher levels than PC12 cells and RIN5-f cells, a rat insulinoma line that is fairly dedifferentiated when compared to RIN 1046-38. Maintaining high PAM activity in RIN 1046-38, similar to maintaining high levels of PC2 and PC3 activity, suggests overexpression of transgenes for amidated peptide hormones such as amylin will result in their efficient production.

Amylin transgenes are efficiently expressed in RIN 1046-38 cells. The rat amylin cDNA was cloned into pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/AMYLIN/IRES/NEO. Expression plasmids similar to this have resulted in good overproduction of other transgenes. Individual stable clones were screened for amylin expression with an *in situ* immunostaining protocol utilizing two dilutions of the primary amylin antibody. At the lower dilution (1:200) all the cells are positive due to the levels of endogenous amylin. At the higher dilution (1:1000), only a subset of clones continued to

stain, presumably due to overexpression of the amylin transgene. Five such clones were picked and shown to express the amylin message. Analysis of polyclones by Northern analysis demonstrates efficient expression of the AMYLIN/IRES/NEO bicystronic message (FIG. 15B).

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Expression of biologically active amidated peptides in RIN 1046-38 cells. Table 4 is a list of known amidated peptide hormones in humans. RIN 1046-38 cells can be engineered to overexpress the cDNA transgenes encoding the precursors to these peptide hormones. RIN lines have been used in the past to express the transgenes for preproglucagon and pancreatic polypeptide resulting in low-level or partial processing to the final amidated polypeptides (Drucker *et al.*, 1986 and Takeuchi *et al.*, 1991). Based on our results with overexpression of human insulin and rat amylin, RIN 1046-38 cells are expected to efficiently process and secrete fully bioactive, amidated polypeptides. As claimed in this patent, this would be done in cells that have also been engineered such that an endogenous gene expressing a secreted protein has been blocked. In this way, a cell overexpressing a biologically active peptide hormone, in this case one that is also amidated, is produced in a defined cellular background for use in *in vitro* large scale production or for *in vivo* cell-based delivery of the active peptide hormone.

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EXAMPLE 7

Human Growth Hormone Production

Methods:

Human growth hormone production plasmid. The gene encoding human growth hormone was isolated on a 2086 base *Bam*HI/*Age*I restriction endonuclease fragment from pOGH (Nichols Institute Diagnostics, Inc., San Juan Capistrano, CA). This fragment corresponds to bases 498 to 2579 of the published gene sequence (SEQ ID NO:9, Seeburg, 1982). The *Bam*HI site is located at the normal site of transcription of the message, 61 bases 5' of the initiator methionine. The *Age*I site is located 3' of the transcribed sequences of the growth hormone gene. This fragment was ligated into pCB6 (Brewer, 1994) that had been digested with *Bg*III and *Age*I, generating pCB6/hGH. The *Bg*III site

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places the hGH gene just downstream of the CMV promoter. The *AgeI* site in pCB6 is located in the human growth hormone polyadenylation element contained in that plasmid. The polyadenylation element is restored by cloning the entire human growth hormone gene into pCB6. Stable transformants of pCB6/hGH are selected in G418.

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Cell culture and stable transfection of cell lines. These studies were performed as described above for insulin producing cells.

Screening and characterization of human growth hormone producing clones.

10 Individual G418 resistant clones generated by electroporation using pCB6/hGH were screened for hGH in the conditioned media using an hGH radioisotopic assay kit (Nichols Institute Diagnostics).

Stimulated growth hormone secretion assay and determination of DNA content and

15 cell number. Done as described for insulin secretion assay and cell number determination.

Results:

Mammalian cell production of human growth hormone.

20 Growth hormone has been shown to be the major regulator of growth in children as well as maintaining or restoring various metabolic functions which can decrease with age (Isaksson *et al.*, 1985 and Arimura, 1994). Purified recombinant human growth hormone is now being produced from mammalian cells in bioreactors for clinical use (Eshkol, 1992). Constitutive cell-based delivery of growth hormone from *ex vivo* engineered primary fibroblasts (Selden *et al.*, 1987 and Heartlein *et al.*, 1994) or primary myoblasts (Dhawan *et al.*, 1991 and Barr and Leiden, 1991) is also being attempted. Fully processed, bioactive growth hormone is produced in all of these systems. Our attempts to engineer neuroendocrine cells to produce recombinant human growth hormone offers two advantages. The first is the ability to engineer high levels of growth hormone into a stable cell line with the various methods outlined here to maximize production levels. This engineering is being done in a cell line in which production of an endogenous secreted protein has been blocked. The

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second advantage is that the growth hormone produced in these cells is packaged into secretory granules where regulated release of growth hormone is possible. Normally, growth hormone is not secreted constitutively, but is secreted in a pulsatile manner as regulated by Growth Hormone Releasing Factor and Somatostatin (Arimura, 1994).

5 Growth hormone produced recombinantly in neuroendocrine cells is known to be secreted through the regulated secretory pathway where its release from the cells can be regulated (Moore and Kelly, 1985). In β -cells, growth hormone produced from a transgene is also secreted via the regulated secretory pathway and secretion can be costimulated along with the endogenous insulin (Welsh *et al.*, 1986).

10 RIN 1046-38 clones produce high levels of recombinant human growth hormone. Seventeen independent clones derived from electroporation of RIN 1046-38 cells with pCB6/hGH were screened for secretion of human growth hormone (hGH). No detectable hGH was detectable from conditioned media from parental RIN 1046-38. Fourteen of the
15 17 clones expressed significant levels of hGH. Six clones were expanded and characterized further.

hGH is expected to be secreted via the regulated secretory pathway in these clones. Cells were cultured for 24 hours in fresh tissue culture media containing 11 mM glucose and 5% fetal calf serum. This conditioned media was collected and immunoreactive hGH
20 was determined (6 independent samples/clone were analyzed, 24 hour collection). Cells were washed and either incubated for one hour in media lacking glucose and containing 100 μ M diazoxide (basal, 2 samples per clone) or incubated for one hour in media containing 5 mM glucose, 100 μ M carbachol, 100 μ M IBMX and amino acids (stimulated,
25 4 samples per clone). Cell numbers for each sample was determined and all hGH values are normalized to μ g of secreted product per million cells. The values are reported in FIG. 14:

30 Over a 24 hour collection, the six clones secreted between 25 and 229 μ g hGH per million cells per 24 hours. Clone EP111/31 has consistently been the highest hGH

producing clone in both the initial screens and in these studies. 229 μ g hGH per million cells per 24 hours is higher than any value of hGH production by a mammalian cell. Previous reported values are in the range of 7-20 μ g/million cells/24 hours (Pavlakakis and Hamer, 1983) and the highest value reported is 40 μ g/million cells/24 hours (Heartlein *et al.*, 1994).

hGH secretion by these six clones is also exquisitely regulated. Basal secretion values were all less than 100 ng/million cells/hour, easily detected in the assay, but barely visible in FIG. 14. Basal values are in the range of 0.1% to 1.0% of the stimulated values for each clone. Stimulated secretion ranged from 6 to 40 μ g hGH/million cells/hour. The one hour output of EP111/31 of 40 μ g/million cells is equivalent to the best 24 hour output reported to date (Heartlein *et al.*, 1994).

The absolute outputs of hGH by RIN clones, as well as the fact that it is secreted via the regulated secretory pathway, are important for both *in vitro* production and *in vivo* cell-based delivery. For *in vitro* production, these cells are producing more hGH in normal tissue culture per 24 hours than previously described cells. Cyclical stimulation of these cells in a bioreactor setting, as previously described for insulin production, can be used for bioreactor production. *In vivo* cell-based delivery of hGH could use the cells in their present form where secretion of hGH would be fairly constant. Alternatively, further engineering of the cells could produce a more physiological pulsatile release of hGH *in vivo* by conferring regulation of growth hormone secretion to growth hormone-releasing factor and/or somatostatin, or other regulators of somatotropes (Arimura, 1994).

EXAMPLE 8

A. Rat Insulin Promoter Factor 1

Methods:

Rat IPF1 expression plasmids. A plasmid containing the rat IPF1 cDNA was obtained from Chris Wright (XB-pdx1). This plasmid contains the open reading frame of rat IPF1 (SEQ ID NO:5, bases 7 to 861) cloned into pXBm (Krieg and Melton, 1984),

placing *Xenopus* β globin 5'- and 3'-transcribed but untranslated sequences 5' and 3' of the rat IPF1 sequence. This construct was made to help stabilize the IPF1 message, allowing for higher steady-state message levels and protein production. A *HinDIII/BamHI* fragment containing the IPF1 and globin sequences was ligated into the *HinDIII* and *BamHI* sites of pCB6 (Brewer, 1994), generating pCB6/IPF1. Alternatively, the IPF1 and globin sequences of pCB6/IPF1 was removed by digestion with *BglII* and *BamHI* and cloned into the *BamHI* site of pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/IPF1/IRES/NEO. Stable transfectants of both of these expression plasmids are selected using G418.

It was not clear that the *Xenopus* β globin sequences would stabilize the IPF1 transgene in RIN cells. For this reason, the IPF1 open reading frame was amplified with the polymerase chain reaction from pCB6/IPF1 using two oligos (GGATCCATGAACAGTGAGGAGCAG, SEQ ID NO:41 and AGATCTTCACCGGGGTTCTGCGG, SEQ ID NO:42). The resulting 867 base product (SEQ ID NO:5) was cloned into pNoTA/T7 (5 Prime to 3 Prime, Inc., Boulder, CO) generating pNoTA/T7/IPF1. The IPF1 open reading frame was removed from pNoTA/T7/IPF1 by digestion with *BamHI* and was ligated into *BamHI* digested pCB6, generating pCB6/IPF1(-Bg). Alternatively, the same IPF1 *BamHI* fragment was ligated into *BamHI* digested pCMV8/IRES/NEO/hGHPolyA, generating pCMV8/IPF1(-Bg)/IRES/NEO. A final expression plasmid was made, ligating the IPF1 *BamHI* fragment into *BamHI* digested pCMV8/Ins3'/IRES/NEO, generating pCMV8/IPF19-Bg/Ins3'/IRES/NEO. The Ins3' nontranslated region in these plasmids was described earlier for the insulin disulfide mutant example and is contained on a 198 base pair *HgaI/HinDIII* fragment. This fragment was ligated into pCMV8/IRES/NEO/hGHPolyA generating pCMV8/Ins3'/IRES/NEO. Stable transfectants of all of these expression plasmids are selected using G418.

Cell culture and stable transfection of cell lines. These studies were performed as described above for insulin producing cells.

Screening and characterization of IPF1 producing clones. Northern analysis of individual G418 resistant clones generated from the various IPF1 expression plasmids was done as described above for the human insulin northern analysis. Blots were hybridized with a ³²P-labeled cRNA probe corresponding to the rat IPF1 open reading frame (SEQ ID NO:5).

Results:

Overexpression of IPF-1 in RIN 1046-38 cells. IPF-1 functions both in the specification of a region of the primitive gut to form pancreas in the maturation of the pancreatic β cells. Because RIN 1046-38 cells retain only some of the differentiated features of a normal b cell, overexpression of IPF-1 in these cells could cause them to function more like mature b cells. Thus redifferentiated RIN cells may serve as a more effective bioreactor for the production of biologically relevant secreted proteins.

In initial experiments, stable transfection of RIN 1046-38 cells with either pCMV8/IPF-1/IRES/NEO or pCB6/IPF-1 resulted in a low number of NEO-resistant colonies. None of these colonies expressed the IPF-1 transgene as demonstrated by Northern blot analysis. A second round of stable transfections were performed with IPF-1 constructs in which the *Xenopus* 5' and 3' betaglobin untranslated sequences (UTR) were removed [IPF-1(-Bg)]. Also, in some constructs, the potentially stabilizing Ins3' UTR was fused immediately downstream of the IPF-1 cDNA. A moderate number of NEO-resistant colonies were obtained from RIN cells transfected with either PCMV8/IPF-1(-Bg)/IRES/NEO or pCMV8/IPF-1(-Bg)/Ins3'/IRES/NEO. Northern analysis of RNA from a mixed population of colonies containing either construct demonstrated that the IPF-1 transgene mRNA was indeed overexpressed related to endogenous IPF-1 (FIG. 16, lanes labeled polyclone #1 and #2). The addition of the 3'Ins UTR to the IPF-1 cDNA did not appear to have a significant effect on IPF-1 transgene expression.

Also shown in FIG. 16 are several clonal RIN lines overexpressing IPF-1 mRNA. As would be expected, some of the clonal lines express more IPF-1 mRNA than the

polyclone and some less since the polyclone represents an average IPF-1 expression level from many drug-resistant colonies. Although not shown here, the polyclonal cells were analyzed for the presence of IPF-1 protein by Western blotting. A slight overexpression of IPF-1 protein was detected over and above endogenously expressed IPF-1 protein in untransfected RIN 1046-38 cells. Clonal lines containing IPF-1 transgenes are currently being analyzed for increased levels of IPF-1 protein.

The IPF-1 containing polyclonal lines were also checked for increased levels of endogenous insulin, glucokinase, and GLUT-2. Increased levels of any one or all three of these proteins could potentially be indicative of more differentiated RIN cells. Northern analysis revealed that neither endogenous insulin nor GLUT-2 mRNA was effected by slight overexpression of IPF-1 protein in the polyclonal RIN lines. However, glucokinase mRNA was slightly elevated in the IPF-1 transgene containing lines. This might be expected since it has been recently demonstrated that IPF-1 interacts with the β -cells glucokinase promoter to play a role in the glucokinase gene regulation (Watada *et al.*, 1996). It is also well proven that IPF-1 is important in insulin gene regulation (Peers *et al.*, 1994), but as stated above, there was not an elevated level of insulin mRNA in the IPF-1 polyclones. Whether or not slight elevation in glucokinase has any physiological significance is currently under investigation. Additionally, some of the clonal lines demonstrating a higher level of IPF-1 mRNA (FIG. 16) than the polyclonal lines are being analyzed in the same manner as the polyclonal RIN lines.

B. Alternative Drug Selection Markers

Methods:

Expression plasmids with alternative selection markers. To facilitate engineering of multiple genes into the same cell line or to optimize expression of a given gene, alternative expression plasmids containing other drug selection markers were designed. The drug selection markers utilized include the hygromycin resistance gene (HYGRO), the puromycin resistance gene (PURO), the dihydrofolate reductase gene (DHFR) conferring resistance to methotrexate, the xanthine-guanine phosphoribosyltransferase gene (GPT)

conferring resistance to mycophenolic acid, the Zeocin resistance gene (ZEO), and the histidinol selection gene (HISD). All of the drug selection genes were tested for their ability to confer drug resistance to RIN cells in two contexts. The first was by substituting the new drug selection gene for the neomycin resistance gene in pCMV8/IRES/NEO. In this context, the drug resistance gene is transcribed off of the CMV promoter as the downstream open reading frame of a bicistronic message. The second is by substituting the new drug selection gene for the neomycin resistance gene in pCB6 (Brewer, 1994) such that the new drug selection gene is driven by the SV40 promoter. pCB7 (Brewer *et al.*, 1994) was constructed this way with the hygromycin resistance gene replacing the neomycin resistance gene.

The open reading frame of the hygromycin resistance gene was amplified using the polymerase chain reaction from pCB7 using oligos (GGGGATCCGATATGAAAAAGCCTG, SEQ ID NO:43 and CGAGATCTACTCTATTCCTTTGC, SEQ ID NO:44). The resulting 1048 base product was digested with *Bam*HI and *Bgl*II and ligated into the *Bam*HI site of pCMV8 generating pCMV8/HYGRO. In a second step, the IRES element (SEQ ID NO:11) contained on a 235 base *Bam*HI/*Bgl*II fragment, was ligated into the *Bam*HI site of pCMV8/HYGRO generating pCMV8/IRES/HYGRO. Stable transformants of pCB7 and pCMV8/IRES/HYGRO are selected using 300 µg/ml hygromycin (Boehringer Mannheim) for 14 days without media changes.

The *E. coli* open reading frame encoding XGPRT was amplified with the polymerase chain reaction from pSV3/GPT (ATCC#37144, Mulligan and Berg, 1980 and 1981) using oligos (CCGGATCCCATGAGCGAAAAAT, SEQ ID NO:45 and GGAGATCTTTAGCGACCGGAGAT, SEQ ID NO:46). The resulting 476 base pair amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/GPT. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/GPT, generating pCMV8/IRES/GPT. The GPT open reading frame was isolated from pCMV8/GPT by digestion with *Bam*HI and *Sma*I

and the resulting 482 base pair fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment and then digested with *Bcl*II, generating pCB8. Stable transformants of pCMV8/IRES/GPT and pCB8 are selected using 2.5 to 3.0 µg/ml mycophenolic acid (Sigma Chemical Co.) in media without exogenous xanthine added for 14 days. Media was changed every 3 to 4 days.

The open reading frame of the mouse dihydrofolate reductase cDNA was amplified with the polymerase chain reaction from pSV3-dhfr (ATCC#37147, Subramani *et al.*, 1981) using oligos (CCGGATCCATGGTTCGACCATTG, SEQ ID NO:47 and GGAGATCTGTTAGTCTTTCTTC, SEQ ID NO:48). The resulting 581 base pair amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/DHFR. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/DHFR, generating pCMV8/IRES/DHFR. The DHFR open reading frame was isolated from pCMV8/DHFR by digestion with *Bam*HI and *Sma*I and the resulting 582 base pair fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment and then digested with *Bcl*II, generating pCB9. Stable transformants of pCMV8/IRES/DHFR and pCB9 are selected using 1 to 10 µg/ml methotrexate (Amethopterin, Sigma Chemical Co.) for 14 days with media changes every 3 to 4 days.

The open reading frame of the HisD gene was amplified with the polymerase chain reaction from pREP8 (Invitrogen, Inc.) using oligos (CCGGATCCATGAGCTTCAATAC, SEQ ID NO:49 and CCAGATCTGCTCATGCTTGCTCC, SEQ ID NO:50). The resulting 1063 base pair amplified product was restricted with *Bam*HI and *Bgl*II and subcloned into the *Bam*HI site of pCMV8, generating pCMV8/HISD. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/HISD, generating pCMV8/IRES/HISD. Stable transformants of pCMV8/IRES/HISD are selected in media with 0.8 to 1.0 mg/ml histidinol for 14 days. Media was changed every 3-4 days.

The puromycin resistance gene was isolated from pPUR (Clontech, Inc.) by digestion with *Pst*I and *Xba*I. The resulting 792 base pair fragment was treated with Klenow fragment and ligated into the *Sma*I site of pCMV8, generating pCMV8/PURO. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/PURO, generating pCMV8/IRES/PURO. The PURO open reading frame was isolated from pCMV8/PURO by digestion with *Nco*I, treated with Klenow fragment, and then digested with *Bam*HI. The resulting 723 base fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment, and then digested with *Bcl*II, generating pCB10. Stable transformants of pCMV8/IRES/PURO and pCB10 are selected using 1.75 to 2.0 µg/ml puromycin (Sigma Chemical Co.) for 10 days with media changes every 3 to 4 days.

The zeocin resistance gene was isolated from pZeoSV (Invitrogen, Inc.) by digestion with *Nco*I and *Acc*I. The resulting 430 base fragment was treated with Klenow Fragment and ligated into the *Sma*I site of pCMV8, generating pCMV8/ZEO. Next, the IRES element (SEQ ID NO:11) was ligated into the *Bam*HI site of pCMV8/ZEO, generating pCMV8/IRES/ZEO. The ZEO open reading frame was isolated from pCMV8/ZEO by digestion with *Rsr*II, treated with Klenow fragment, and then digested with *Bam*HI. The resulting 406 base fragment was ligated into pCB6/intron (see above) that had previously been digested with *Nar*I, treated with Klenow fragment, and then digested with *Bcl*II, generating pCB11. Stable transformants of pCMV8/IRES/ZEO and pCB11 are selected using 400 µg/ml Zeocin (Invitrogen, Inc.) for 14 days with media changes every 3 to 4 days.

EXAMPLE 9

GLUT-2 and Glucokinase Expression

Methods:

Rat GLUT-2 expression plasmids. Construction of pCB7/GLUT-2 containing base pairs -108 to +1835 of the rat GLUT-2 cDNA has been described previously (Hughes, et

al., 1992). In addition, this same fragment of the GLUT-2 cDNA was ligated into pCB7/intron to generate pCB7/intron/GLUT2.

Glucokinase expression plasmids. The cDNA encoding the islet isoform of glucokinase was isolated as a 1763 bp fragment corresponding to bp 180 to 1927 of the published sequence (Hughes, *et al.*, 1991) and cloned into the XbaI site of pCB7 to generate pCB7/GK. Alternatively, the same 1763 bp XbaI fragment was cloned into the XbaI site of pCMV8/IRES/GPT/hGH poly A to generate pCMV8/GK/IRES/GPT.

Glucokinase Western blot. The level of glucokinase protein in RIN cell lines was measured by Western blot hybridization analysis, using antibody β GK-1 prepared against a glucokinase/glutathione-S transferase fusion protein (Becker, *et al.*, 1996). Cell lysates were prepared by suspending cells in ice-cold buffer consisting of 20 mM K_2HPO_4 , 1 mM EDTA, and 110 mM KCl, and sonicating cells on ice (3 bursts, Sonic Dismembrator 50, Fisher Scientific). Supernatants were prepared by centrifugation at 14-16,000 x g in a refrigerated microcentrifuge. Protein concentration in the supernatant was determined by the method of Bradford (1976) and 5 μ g of protein was suspended in an equal volume of 2 x sample buffer (100 mM Tris, 4% SDS, 0.2% bromphenol blue, 20% glycerol, 10% β -mercaptoethanol, pH 6.8), heated at 95°C for 5 minutes and electrophoresed using 8-16% Tris-glycine gels (Novex, San Diego, CA). Proteins were transferred to PVDF membranes (BioRad) and blocked with 4% dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0). The blot was incubated overnight with antibody β GK-1 diluted 1:10,000 in TBST + 1% BSA and bands visualized by incubation with an alkaline phosphatase-conjugated second antibody (sheep anti-rabbit, Sigma) and nitroblue tetrazolium with bromochloro-indoyl phosphate.

Glucokinase and Hexokinase Enzymatic Activity. Glucose phosphorylation was measured in cell homogenates by following the conversion of U- ^{14}C glucose to U- ^{14}C glucose-6-phosphate as previously described (Kuwajima, *et al.*, 1986). Glucokinase and hexokinase activities were discriminated by performing the assay in the presence or

absence of 10 mM glucose-6-phosphate, an inhibitor of low K_m hexokinase activity (Wilson, 1985).

Results:

5 Iterative Engineering of RIN Cell Lines for GLUT-2, Glucokinase, and Human Proinsulin Expression. Having demonstrated the feasibility of iterative engineering of a single gene (human proinsulin), the inventors sought to determine if similar strategies would be applicable to expression of several different genes in RIN 1046-38 cells. The inventors began with an initial round of transfection that produced clones selected with
10 neomycin and overexpressing human proinsulin, as shown by the appearance of a band corresponding to human proinsulin in the primer extension analysis depicted in FIG. 17. Cells expressing the human proinsulin gene (the R5C.I-17 line described above) were then transfected with a plasmid in which the CMV promoter is used to direct expression of the cDNA encoding the rat islet isoform of glucokinase and the GPT resistance gene coupled
15 to glucokinase by an IRES element (pCMV8/GK/IRES/GPT). The increased glucokinase expression is shown for cell line EP 23/31 selected with mycophenolic acid (FIG. 17). Finally, the GLUT-2 gene was introduced using the pCB7/intron/GLUT-2 plasmid, which contains a hygromycin resistance gene and which utilizes the CMV promoter to drive expression of the GLUT-2 cDNA. The resultant "triple positive" cell lines are represented
20 by line EP 49/206 in FIG. 17. The data of this FIG. 19 clearly demonstrate the feasibility of stable iterative introduction of three different genes in RIN cells. The human insulin transgene in R5C.I-17 cells has been expressed stably for over one year of continuous cell culture, and as shown in FIG. 17, is maintained stably through two rounds of transfection with other genes. The glucokinase and GLUT-2 transgenes have been stably expressed for
25 50 population doublings in continuous culture, and none of the three transgenes require addition of antibiotics for maintenance of stable expression.

In EP 49/206 cells both GLUT-2 and human proinsulin transgenes are readily detected, while glucokinase transgene expression is relatively low (FIG. 17). Therefore,
30 R5C.I-17 cells, which express human insulin, were transfected with pCB7/GK and several

new clones with stronger expression of glucokinase were isolated. The resulting EP 40/110 and EP 40/107 cell lines exhibit a marked increase in glucokinase mRNA compared to the R5C.I-17 cells from which they are derived (data not shown). EP 40/107 cells were injected into nude rats and were explanted after 15 and 22 days *in vivo*.

5 Northern blot analysis demonstrates stable maintenance of the highly expressed glucokinase transgene during *in vivo* passage in these cells, in a manner very similar to the insulin and GLUT2 transgene.

Measurements of glucokinase protein and enzymatic activity. In order to evaluate
10 the levels of glucokinase expression in the various lines in more detail, glucokinase protein and enzymatic activity in the parental and two transfected lines were measured. FIG. 18 shows Western blot analysis of glucokinase protein in RIN 1046-38, R5C.I-17, EP 49/206, and EP 40/110 cells, using antibody β GK-1 raised against a glucokinase/glutathione-S-transferase fusion protein (Becker, et al., 1996). EP 49/206
15 cells contain approximately 50% more, and EP 40/110 cells approximately ten times as much glucokinase protein as the RIN 1046-38 or R5C.I-17 cell lines. These changes in glucokinase protein levels are well correlated with estimates of glucokinase enzymatic activity in extracts of these cell lines.

20 Thus, RIN 1046-38 and R5C.I-17 cells contain 29 and 34 U/g protein of glucose phosphorylating activity, respectively, when assayed at 20 mM glucose in the absence of glucose-6-phosphate, but this activity is reduced to 2.0 and 1.3 U/g, respectively, when the assay is conducted in the presence of 10 mM glucose-6-phosphate, indicating that more than 90% of the glucose phosphorylating activity of these lines is contributed by low K_m ,
25 glucose-6-phosphate-sensitive hexokinases (FIG. 19).

EP 49/206 cells have a similar level of glucose phosphorylating capacity as RIN 1046-38 or R5C.I-17 cells when measured in the absence of glucose-6-phosphate (FIG. 19). In the presence of glucose-6-phosphate, EP 49/206 cells contain 3.6 U/g of glucose
30 phosphorylating activity, approximately double the level found in the other two cell lines

(FIG. 19), consistent with the modest enhancement in immunodetectable glucokinase protein in the EP 49/206 line.

Finally, EP 40/110 cells contain slightly more than double the total glucose phosphorylating activity of the other cell lines (63 U/g), and exhibit a more than 10-fold enhancement in activity in the presence of glucose-6-phosphate (27.3 U/g). Thus, in EP 40/110 cells, hexokinase activity represents approximately 57% of the total glucose phosphorylating capacity, with the remainder contributed by glucose-6-phosphate-insensitive glucokinase.

EXAMPLE 10

Regulation Of Insulin Secretion From Engineered Insulinoma Cell Lines

Methods:

Human Islets. Human pancreatic islets were isolated by Dr. Camillo Ricordi, University of Miami, cultured in CMRL-1066 with 10% FBS overnight, and then shipped to Dallas by overnight express. Immediately upon receipt (approximately 36 hours after islet isolation), a portion of the islets, usually 10,000-16,000 islet equivalents (EIN), were washed in 10 ml PBS and pelleted. The islet pellet was extracted by sonication in 1 M acetic acid-0.1% BSA (RIA grade, Fraction V; Sigma Chemicals). The remaining islets were cultured for 1 week for stimulated secretion assays at 1000 EIN/well in 6 well dishes, under the same conditions as the engineered cell lines (see above), except the glucose concentration was 6 mM.

Insulin Secretion Measured in Static Incubation Assays. Cells were plated in 12-well dishes (Corning Glass Works, Corning, NY) at a density of approximately 250,000 cells per well and allowed to grow for 48 hours. Cells were washed twice for 20 minutes each in HEPES/bicarbonate buffered salt solution (HBBSS; 114 mM NaCl, 4.7 mM KCl, 1.21 mM KH_2PO_4 , 1.16 mM MgSO_4 , 25.5 mM NaHCO_3 , 2.5 mM CaCl_2 , 10 mM Hepes) with 0.1% bovine serum albumin but without glucose. Secretion studies were then conducted by incubating cells in HBBSS containing 0.5% BSA and with glucose in a

range of concentrations from 0 to 20 mM for two hours. These experiments were conducted in the presence and absence of 2 mM 5-thiogluco-
se (Sigma, St. Louis, MO) and in the presence or absence of 100 μ M isobutylmethylxanthine (IBMX). Following incubation, media was collected and assayed for insulin by radioimmunoassay with the
5 DPC method, as described above. Secretion was normalized for cell number by measuring total protein in each well by the method of Bradford (1976), using the kit from Bio-Rad, Hercules, CA.

Insulin Secretion Measured by Perifusion. Cells were grown on Fibracel discs
10 (New Brunswick Scientific) in 250 ml spinner flasks (Techne, Cambridge, MA) at an initial density of 0.5×10^6 cells/disc, in the same medium used for tissue culture. Approximately 30 discs with adherent cells were transferred to a 10 cm x 10 mm (inner diameter) Pharmacia column containing tissue culture medium, with the discs occupying approximately 2 ml of the column bed. Cells were perifused at a flow rate of 0.7 ml/min,
15 beginning with a 30 minute wash with HBBSS, 0.5% BSA lacking glucose. The same medium was perifused for the first 45 minutes of sample collection (basal period), with samples taken at a rate of 2 minutes/tube. The perfusate was then switched to HBBSS, 0.5% BSA containing 10 mM glucose + 100 μ M IBMX for 45 minutes, and then returned to the basal medium for a final 45 minute period. Samples were collected and subjected to
20 insulin radioimmunoassay.

Glucose Usage Measurements. Glucose usage was monitored by administration of 5- 3 H glucose to intact cells as described (Becker, et al., 1996 and Hughes, et al., 1993) with some modifications. Cells were grown to approximately 75% confluence in 24-well
25 dishes (Corning Glass Works, Corning, NY) in medium 199, supplemented as described above, and then washed twice in HBBSS, 0.1% BSA lacking glucose for 20 minutes each. Thereafter, cells were preincubated in 250 μ l HBBSS, 0.1% BSA with either 1 or 20 mM glucose for 10 minutes, followed by initiation of the glucose usage period by addition of tracer 5- 3 H glucose to a specific activity of 2 μ Ci/ μ mol. After 30 minutes, the reactions
30 were stopped by addition of 100 μ l of ice-cold 10% TCA. After complete lysis of the

cells, the suspension was centrifuged at 14,000 x g for 10 minutes and the supernatant used to determine $^3\text{H}_2\text{O}$ water production as previously described, after correction for the efficiency of equilibration with a $^3\text{H}_2\text{O}$ standard (Hughes, et al., 1993).

5 **Results:**

Basal Insulin Secretion and Insulin Content. The insulin content of the four cell lines was measured by an assay that detects both rat and human insulin (DPC), using a human insulin standard curve (Table 8). Using this method, RIN 1046-38 cells were found to contain 34.5 ± 2.4 ng insulin/6 μg DNA, while R5C.I-17, EP 49/206, and EP 40/110
10 cells exhibited 11.7-, 7.5-, and 5.2-fold increases in insulin content, respectively (note that 6 μg DNA is equivalent to 1×10^6 cells). Reassay of a subset of these samples using by the Linco method that is specific for human insulin found no detectable insulin in RIN 1046-38 cells, while the R5C.I-17, EP 49/206, and EP 40/110 cell lines contained 209, 126, and 115 ng/6 μg DNA, respectively, demonstrating good agreement in terms of the
15 relative levels of insulin contained in the three engineered cell lines.

TABLE 8.

<u>Cell Line</u>	<u>Insulin Content(ng/6 μg DNA)</u>
RIN 1046-38	34.5 ± 2.4
$\beta\text{G I/17}$	410.4 ± 16.8
$\beta\text{G 49/206}$	262.0 ± 10.5
$\beta\text{G 40/110}$	179.2 ± 13.3

Table 8. Insulin Content in RIN Cell Lines. Insulin content was measured as described in Materials and Methods. Values represent the mean \pm S.E.M. for 5
20 independent determinations per cell line.

 Basal insulin secretion (in the absence of secretagogues) was measured from unmodified RIN 1046-38 cells, and the engineered lines R5C.I-17, EP 49/206, and EP 40/110. As shown in FIG. 20, stable transfection of RIN 1046-38 cells with the human proinsulin gene to generate line R5C.I-17 caused a 5.5-fold increase in basal insulin
25

secretion (from 3.1 ± 0.7 ng/mg protein/2 h to 27.2 ± 8.4 ng/mg protein/2 h). Stable expression of the GLUT-2 and glucokinase genes (line EP 49/206) or high level expression of the glucokinase gene alone (line EP 40/110) caused a 66% and 80% decrease in basal insulin secretion, respectively (to 5.8 ± 1.7 ng/mg protein/2 h and 3.5 ± 0.5 ng/mg protein/2 h, respectively). Two other clones termed EP 49/216 and β G 40/107 derived from the same transfections that produced EP 49/206 and EP 40/110, respectively, and expressing similar levels of GLUT-2 and glucokinase, exhibited a similar decrease in basal insulin secretion relative to R5C.I-17. The R5C.I-17 cell line from which EP 49/206 and 216 and EP 40/107 and 110 are derived is a pure clone and has exhibited complete stability of insulin content for a year of continuous cell culture. Thus, transfection of R5C.I-17 cells with GLUT-2 and/or glucokinase appears to cause a lowering of insulin content, possibly via a partial reduction in insulin stores in cells cultured in media containing 11 mM glucose. This decrease in content may contribute to the decline in basal insulin production noted in the multiply engineered lines, but is not sufficient to fully account for the observed effects, suggesting that expression of GLUT-2 and/or glucokinase also works by maintaining a lower basal insulin secretion in these cells.

Secretagogue-Induced Insulin Secretion. The inventors next evaluated the effect of two well known β -cell secretagogues, glucose and IBMX, on the same cell lines used for measurement of basal insulin secretion. As shown in FIG. 21, unengineered RIN 1046-38 cells of relatively low passage number (passage 15) exhibited a 2.8 ± 1.1 -fold increase in insulin secretion in response to 5 mM glucose and a 3.8 ± 0.7 -fold increase in insulin secretion in response to 5 mM glucose + IBMX relative to cells incubated in the absence of secretagogues. Insulin secretion in response to glucose alone was not enhanced in R5C.I-17 cells compared to RIN 1046-38 cells, but was significantly increased in response to glucose + IBMX, to a value of 6.5 ± 0.5 -fold above unstimulated levels. The responses to glucose alone and to glucose + IBMX were significantly enhanced in both the EP 49/206 and EP 40/110 cells compared to either RIN 1046-38 or R5C.I-17 cells. Insulin secretion was stimulated by 6.1 ± 1.8 and 7.6 ± 1.7 -fold in response to 5 mM glucose alone, and by 10.8 ± 2.8 -fold and 15.1 ± 4.3 -fold in response to glucose + IBMX from EP

49/206 and EP 40/110 cells, respectively. Similar findings were made for the sister cell lines EP 49/216 and EP 40/107 (data not shown). When stimulated with glucose + IBMX, RIN 1046-38 cells secreted 11.5 ± 2.1 ng insulin/mg cellular protein/2 h, while R5C.I-17, EP 49/206, and EP 40/110 cells secreted 109 ± 7.6 , 62.5 ± 16.1 , and 52 ± 14.8 ng insulin /mg cellular protein/2 h, respectively. These data show that all three engineered lines have an enhanced capacity for insulin secretion relative to the unengineered RIN 1046-38 cells.

The further enhancement in fold-response in the EP 49/206 and EP 40/110 cells, however, appears to be mainly due to the GLUT-2 and glucokinase transgene-mediated suppression of basal insulin secretion shown in FIG. 20.

Dynamics of Insulin Secretion. The foregoing results on insulin secretion from the various cell lines were achieved by static incubation studies in which cells were incubated for 2 hours after application of the secretagogues. To learn whether the robust responses of the engineered lines to glucose and IBMX occur with appropriate dynamics, perfusion studies on the same four cell lines used for static incubation measurements were performed (see FIG. 21). As shown in FIG. 22, parental RIN 1046-38 cells exhibit a sluggish and small (approximately 2-fold) response when the perfusion lacking secretagogues is switched to one containing 10 mM glucose + 100 μ M IBMX. In contrast, R5C.I-17 cells respond rapidly, such that the maximal response of approximately 15-fold above the baseline is reached within 10 minutes. However, in these cells, the response subsequently declines rapidly, and is sustained at a level of only 4-6 fold above baseline during the last half of the stimulation period. Finally, EP 49/206 and EP 40/110 cells exhibit a similarly large and rapid response to glucose + IBMX as observed for R5C.I-17 cells, but insulin secretion is now sustained at 15-fold above background for most of the stimulatory period. When EP 49/206 and EP 40/110 cells are switched back to perfusion buffer lacking secretagogues, an initial paradoxical burst of insulin secretion is observed followed by a rapid return to the original baseline. It should be noted that similar spikes of insulin secretion have been observed in response to removal of a secretory stimulus in islet perfusion or pancreas perfusion experiments (Becker, et al., 1994 and Ogawa, et al., 1992).

The data in FIG. 22 are normalized to basal insulin secretion (occurring in the absence of secretagogues) for each cell line. As reported for the static incubation studies (see above) both basal insulin secretion and the absolute amount of insulin secreted from perfused R5C.I-17 cells in response to stimulation was greater than for the EP 49/206 and EP 40/110 lines (data not shown). However, when the data are expressed in terms of fold-response as in FIG. 22, the latter two cell lines exhibit a sustained response to stimulation that is not apparent in the control cells, indicating that expression of GLUT-2 and/or glucokinase provides better control of insulin secretion than in cells containing only the human proinsulin transgene.

Glucose Dose Response in the Presence and Absence of 5-thiogluco- The inventors have previously shown that cell lines stably transfected with GLUT-2 that undergo a spontaneous increase in glucokinase activity exhibit maximal stimulated insulin secretion at approximately 50 μ M glucose, but that this response can be shifted to a maximum at 2-5 mM glucose by preincubation of the cells with 40 mM 2-deoxyglucose (Ferber, et al., 1994). To perform similar studies on the new engineered cell lines the inventors used 5-thiogluco-, a glucose analog that is a more potent inhibitor of hexokinase than 2-deoxyglucose (Wilson and Chung, 1989), allowing the studies to be performed with relatively low amounts of 5-thiogluco- (2 mM) present throughout the secretion assay rather than as a preincubation.

Glucose dose-response studies were performed on each of the four cell lines used in the earlier experiments (FIG. 23). IBMX was included throughout in order to allow clear analysis of the otherwise poorly responsive unengineered RIN 1046-38 cells. In the absence of 5-thiogluco-, EP 49/206 cells were distinct from the other three lines in that they exhibited a significant response to the lowest concentration of glucose tested (0.05 mM) (FIG. 23C). All four lines exhibited maximal responses to glucose at a concentration of 0.25 mM, although in other experiments not shown here lines expressing high levels of glucokinase such as EP 40/110 and EP 40/107 were sometimes observed to

exhibit a further doubling of insulin secretion as glucose was raised from 1 to 3 mM. The maximal fold-responses to glucose + IBMX in FIG. 23 were similar to those reported above (FIG. 21), being 5.3 ± 0.3 -fold for RIN 1046-38 cells, 5.7 ± 0.7 -fold for R5C.I-17 cells, 12.7 ± 0.8 -fold for EP 49/206 cells, and 12.7 ± 1.7 -fold for EP 40/110 cells.

Inclusion of 2 mM 5-thiogluco-
se in the secretion buffer generally caused a shift in
glucose dose-response for all four cell lines, but with a different pattern for each line. In
RIN 1046-38 cells, the effect was modest, consisting of a 50% reduction in insulin
secretion at 0.25 mM glucose, such that maximal secretion was now achieved at 0.5 mM
glucose (FIG. 23A). The high basal insulin secretion occurring from R5C.I-17 cells in the
absence of secretagogues or in the presence of IBMX alone was suppressed by
approximately 50% by inclusion of 5-thiogluco-
se. In addition, inclusion of 5-thiogluco-
se caused a shift in glucose stimulation in R5C.I-17 cells such that the first response was
observed at 0.5 mM, and the maximal response, which was less than in the absence of 5
thiogluco-
se (3.9-fold versus 5.7-fold), was seen at 1 mM glucose (FIG. 23B). As shown in
FIG. 23C, 5-thiogluco-
se had no effect on insulin secretion from EP 49/206 in the absence
of glucose, but completely eliminated the response to 0.05 and 0.25 mM glucose, resulting
in a glucose-dose response curve closely resembling that of R5C.I-17 cells, except that the
fold-response was clearly larger, consistent with the findings of FIG. 20. EP 40/110 cells
exhibited the most normalized glucose response curve in the presence of 5-thiogluco-
se (FIG. 23D). The analog completely eliminated the response to 0.25 mM glucose and
reduced the response to 0.5 mM glucose by 70%. In addition, progressively increasing
responses to 1, 3, and 5 mM glucose were observed, with a return to the same maximum
stimulation as observed in the absence of 5-thiogluco-
se occurring at 5 mM glucose. Thus,
cell lines with high levels of glucokinase expression such as EP 40/110 (and EP 40/107,
for which similar results were obtained, data not shown) not only exhibit the largest
insulin secretion responses to glucose or glucose + IBMX, but also appear to be the most
amenable for correction of glucose dose responsiveness via inhibition of low K_m
hexokinase activity.

Note that the data of FIG. 23 are highly consistent with those in FIG. 20, despite the fact that they were carried out as completely independent experiments. This is in keeping with our general observation that the secretory phenotype of the engineered cell lines was stably maintained throughout the course of these studies (a period of six months, or roughly 40 population doublings).

Glucose Usage. In order to determine if the different cellular phenotypes observed in the foregoing experiments were correlated with the capacity for glucose metabolism, the $5\text{-}^3\text{H}$ glucose usage at low (1 mM) and high (20 mM) glucose was measured. As shown in FIG. 24, EP 49/206 cells were distinct from the other three lines in two ways. First, these cells exhibited a clearly higher rate of glucose usage at both 1 mM and 20 mM glucose than the other three cell lines. Second, inclusion of 5-thiogluconase during the glucose usage assay had a potent inhibitory effect on glucose usage at 1 mM glucose in the EP 49/206 cells, but not in the other cell lines. The major difference between EP 49/206 cells and the other three lines is their high level of GLUT-2 expression, suggesting that overexpression of the transporter might be responsible for the enhanced glucose usage in these cells. EP 40/110 cells, which express high levels of glucokinase, had similar rates of glucose usage at low or high glucose as the unengineered RIN 1046-38 cells or R5C.I-17 cells containing only the human proinsulin transgene. The high rate of glucose usage at low glucose in EP 49/206 cells is accompanied by a unique response to the lowest concentration of glucose tested in insulin secretion studies (0.05 mM), as shown in FIG. 23.

EXAMPLE 11

Genomic Site-Directed Mutagenesis with Oligonucleotides

The inventors have previously demonstrated that derivative cell lines of the RIN 1046-38 cell line are capable of performing homologous recombination by disrupting an allele of the hexokinase I gene. Feasibility studies are currently underway to determine if RDOs or DNA oligonucleotides can be used for the purpose of targeted gene disruption in RIN and other cell lines. Two test systems have been designed for testing

oligonucleotides: the disruption of the neomycin phosphotransferase transgene, and the disruption of the glucose transporter, type 2 (GLUT-2). As a preliminary experiment to testing RDOs or DNA oligonucleotides, protocols for efficient delivery of DNA into RIN cell lines by electroporation have been optimized.

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A. Optimization of transfection of RIN cell lines.

A number of transfection protocols were tested on RIN 1046-38 cell lines including a variety of electroporation conditions and multiple kinds of liposome-mediated transfection. All protocols, except one set of electroporation conditions, failed to produce transfection efficiencies of greater than 5%. Protocols were optimized for delivery of exogenous DNA to RIN cells by electroporation using two types of DNA: a plasmid vector encoding beta-galactosidase (β -gal) that is transcribed from the CMV promoter, and a DNA oligonucleotide (62mer) that had been radiolabeled with ^{32}P -dCTP. One set of electroporation conditions resulted in 25 - 40% transfection of the total cell population as determined by colorimetric, cytochemical assays for β -gal activity (Bassel-Duby *et al.*, 1992). Cells were grown to about 80% confluence in Medium 199/ 5% fetal calf serum/ 11 mM glucose (Growth Medium) and were re-fed with fresh Growth Medium one day prior to electroporation. Cells were harvested by trypsinization, counted, pelleted by centrifugation at 1000 rpm for 5 minutes, and resuspended in Growth Medium at a density of 2×10^7 cells/ml. 0.5 ml of cell suspension was mixed with 60 μl of the following DNAs: either 10 μg of β -gal plasmid or 40 nM of oligonucleotide and 110 μg of sonicated salmon sperm DNA. The cells plus DNA were mixed gently, transferred to a 0.4 mM cuvette, and electroporated at 600 μF , 250 volts using and Electro Cell Manipulator 600, BTX Electroporation System. The electroporated cells were removed from the cuvette and diluted into 25 - 30 mls of 37°C Growth Medium containing 5 mM butyrate. Following incubation for 12-16 hours at 37°C, 5% CO_2 in the growth medium with butyrate, cells were washed once with growth medium, and maintained in growth medium. In the case of cells transfected with β -gal, cells were maintained 48-72 hours following transfection and fixed with 0.5% glutaraldehyde for 10-15 minutes for

cytochemical detection of β -gal using the 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (x-gal) as a substrate (Bassel-Duby *et al.*, 1992).

To determine if conditions optimized for plasmid DNA would translate to efficient uptake of oligonucleotides, the above electroporation protocol was applied to a 62mer DNA oligonucleotide that had been radiolabeled with ^{32}P using the Redi-prime Random Primer labeling Kit (Amersham Life Sciences). Oligonucleotide (40 nM) was electroporated into cells. Cells were analyzed post-transfection at 0, 3, 6, and 24 hours in two ways. First, total radioactivity in the media, cytoplasmic cellular fractions, and nuclear cellular fractions was determined by scintillation counting. And second nucleic acids were harvested from cellular fractions by phenol/chloroform/isoamyl extraction and fractionated through denaturing polyacrylamide (PAG) gels (Ebbinghaus *et al.*, 1996).

There was a marked enhancement in nuclear radioactivity in the presence of electroporation as compared to control cells that were mixed with oligonucleotide but not electroporated. In the presence of electroporation, about 29, 55, and 66% of total intracellular counts segregated to the nuclear fraction at 0, 3, 6, and 24 hours, respectively.

In contrast, only 1 - 24% of total intracellular radioactivity was detected in the nuclear fraction through the 24 hour time point. It was also observed that intact, apparently full-length oligonucleotide could be extracted from cells which had been electroporated, as evidenced by fractionation on denaturing PAG gels and autoradiography. Extracts from cells that had been mixed with the oligonucleotide but not electroporated did not yield detectable oligonucleotide by this method of analysis suggesting that radioactivity that was detected in the non-electroporated cellular fractions was derived from the exchange of radiolabel, not from the oligonucleotide.

From these studies it has been concluded that the electroporation protocol described above is a preferred method for transfecting both plasmid DNA and oligonucleotides into RIN cells.

B. Disruption of the neomycin phosphotransferase (NPT) transgene by RDOs.

Multiple RIN cell lines are available that have been engineered to contain an integrated copy of the NPT gene. An RDO for disruption of transgenic NPT has been designed that is complementary to nucleotides to 54 to 78 of NPT counting the "A" of the first methionine as 1. Further, the RDO contains a single base change relative to the wild-type NPT (A to C at position 66). If gene conversion by the RDO is successful, a T will be converted to a G, Tyr22 will be converted to a stop codon, resistance to G418 will be lost, and a unique *Mae* I restriction site will be introduced. The RDO also contains features previously described such as self-annealing hairpin loops at each end, and 2'-O-methylation of the ribose sugars. The sequence of the RDO with these features is (5' to 3' and referred hereafter as AT142):

GCTATTCGGCTAGGACTGGGCACAATTTTuugugcccagTCCTAgccgaauagcGCGC
GTTTTCGCGC (SEQ ID NO:51), where large caps represent DNA residues and small, bold letters indicate RNA residues.

RIN cell lines with a single integrated copy of NPT will be electroporated, as described in materials and methods, with varying concentrations of RDO AT142. 4 to 6 hours following transfection genomic DNA from pools of transfectants will be analyzed for detection of a T to G conversion at position 66 of the NPT transgene. Following isolation of genomic DNA, the first about 200 base pairs of the NPT transgene will be amplified by the polymerase chain reaction (PCR) using oligonucleotides that flank position 66. Following amplification, PCR products will be digested with *Mae* I to determine if any gene conversions have occurred. If the case of successful gene inactivation by the RDO, the PCR product will be digested into two bands. The wild-type NPT transgene PCR product will be resistant to *Mae* I digestion. If NPT gene disruption is detectable by PCR/*Mae* I digestion, small pools of clones will be analyzed for loss of resistance to G418. Following electroporation, cells will be plated into 96 well plates at

densities of 3 to 5 cells/well. 3 days following electroporation, cells will be exposed to G418, and each well will be scored for the presence of cell death.

C. Disruption of transgenic GLUT-2 in RIN and 293 cell lines.

RIN cell lines and 293 cell lines have been engineered to express high levels of a transgenic GLUT-2 transporter as detailed herein above. The presence of this transporter confers sensitivity to the cytotoxin streptozotocin (STZ), and thereby provides a means of negative selection (Schnedl *et al.*, 1994). Both RIN and 293 cell lines that express high levels of a GLUT-2 transporter will be transfected with RDOs designed to target and disrupt transgenic GLUT-2, and 4 - 6 hours later cells will be exposed to cytotoxic levels of STZ. Surviving clones will be analyzed for the presence of an inactivated GLUT-2 transgene by analysis of genomic DNA. In the case of the targeted inactivation of transgenic GLUT-2, leucine at position 10 will be converted to a stop codon as a result of a T to A conversion, and a unique *Avr* II restriction site will be created in the transgenic GLUT-2. This unique site can be detected by the amplification of genomic DNA that flanks the site by PCR, followed by digestion of the amplified DNA with *Avr* II. One such RDO that potentially accomplishes the targeted disruption as described above is the following sequence:

TCACCGGAACCTAGGCTTTCACCTGTTTTT**acaguga**aaagCCTAG**guuccgguuga**GCGC
GTTTTCGCGC (SEQ ID NO:52), where large capitals represent DNA residues and small bold letters represent RNA residues.

Attempts to disrupt transgenic GLUT-2 will also be made with non-chimeric DNA oligonucleotides that contain phosphorothioate modified backbones to enhance stability. It has been reported that inclusion of phosphorothioate derivatives within the DNA backbone decreases sensitivity to nucleases (Vosberg and Eckstein, 1982; Monia *et al.*, 1996). Oligonucleotides have been designed that should selectively target the transgenic GLUT-2 by spanning an area of homology that is interrupted in the endogenous GLUT-2 gene by an intron. If targeting and modification of the GLUT-2 transgene are successful,

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glutamine at position 35 will be converted to a stop codon, and a new Afl II site will be introduced into the DNA at this position. Four DNA oligonucleotides will be examined for the ability to target and disrupt the transgenic GLUT-2: oligo name: AT157 (5'to3')

5 GsGTTCCCTTCCAGTTCGGATATGACATCGGTGTGATCAATGCACCTTAAGAGG
TAATAATATCCCATTATCGACATGTTTTGGGTGTTTCCTsC (SEQ ID NO:53),

oligo name: AT158 (5' to 3')

GsAGGAACACCCAAAACATGTCGATAATGGGATATTATTACCTCTTAAGGTGC
ATTGATCACACCGATGTCATATCCGAACTGGAAGGAACsC (SEQ ID NO:54),

oligo name: AT159 (5' to 3')

10 GsGATATGACATCGGTGTGATCAATGCACCTTAAGAGGTAATAATATCCCATT
ATCGACATsG (SEQ ID NO:55), and oligo name: AT160
CsATGTCGATAATGGGATATTATTACCTCTTAAGGTGCATTGATCACACCGAT
GTCATATCsC (SEQ ID NO:56).

Each of above the 4 oligonucleotides have phosphorothioate modifications in the
15 backbone near the 3' and 5' ends as indicated by "s" in the sequence.
Oligonucleotides will be introduced into cells both as single-stranded molecules and as
double-stranded complexes. The following oligonucleotide pairs contain complementary
sequences and will form duplexes: AT157-AT158, AT157-AT160, AT158-AT159, and
AT159-AT160. Cell lines that express high levels of transgenic GLUT-2 will be
20 electroporated with oligonucleotides as described above, and exposed to levels of STZ
that are lethal to cells expressing non-disrupted transgenic GLUT-2. Genomic DNA of
surviving cells will be analyzed for the presence of disrupted transgenic GLUT-2 by
amplification of DNA containing the putative mutation by PCR, followed by digestion
with Afl II.

25 * * *

All of the compositions and/or methods disclosed and claimed herein can be made
and executed without undue experimentation in light of the present disclosure. While the
compositions and methods of this invention have been described in terms of preferred
embodiments, it will be apparent to those of skill in the art that variations may be applied
30 to the compositions and/or methods and in the steps or in the sequence of steps of the

method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and
5 modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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WO 91/10470

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Board of Regents, The University of Texas
System

(B) STREET: 201 West 7th Street

(C) CITY: Austin

(D) STATE: Texas

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 78701

(A) NAME: Betagene, Inc.

(B) STREET: 2600 Stemmons Freeway, Suite 125

(C) CITY: Dallas

(D) STATE: Texas

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 75207

(ii) TITLE OF INVENTION: RECOMBINANT EXPRESSION OF PROTEINS FROM
SECRETORY CELL LINES

(iii) NUMBER OF SEQUENCES: 56

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: Unknown

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/589,028

(B) FILING DATE: 19-JAN-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 515 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCGGG GGTCTTCTG CCATGGCCCT GTGGATGCGC CTCCTGCCCC TGCTGGCGCT 60
GCTGGCCCTC TGGGGACCTG ACCCAGCCGC AGCCTTTGTG AACCAACACC TGTGCGGCTC 120
ACACCTGGTG GAAGCTCTCT ACCTAGTGTG CGGGGAACGA GGCTTCTTCT ACACACCCAA 180

194

GACCCGCCGG GAGGCAGAGG ACCTGCAGGT GGGGCAGGTG GAGCTGGGCG GGGGCCCTGG 240
 TGCAGGCAGC CTGCAGCCCT TGGCCCTGGA GGGGTCCCTG CAGAAGCGTG GCATTGTGGA 300
 ACAATGCTGT ACCAGCATCT GCTCCCTCTA CCAGCTGGAG AACTACTGCA ACTAGACGCA 360
 GCCCGCAGGC AGCCCCCCAC CCGCCGCCTC CTGCACCGAG AGAGATGGAA TAAAGCCCTT 420
 GAACCAGCAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAAC CCCCCCCCCC 480
 CCCCCTGCAG CAATGGCAAC AACGTTGCGG AATTC 515

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 110 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met	Ala	Leu	Trp	Met	Arg	Leu	Leu	Pro	Leu	Leu	Ala	Leu	Leu	Ala	Leu	1	5	10	15
Trp	Gly	Pro	Asp	Pro	Ala	Ala	Ala	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	20	25	30	
Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	35	40	45	
Phe	Tyr	Thr	Pro	Lys	Thr	Arg	Arg	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	50	55	60	
Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	65	70	75	80
Ala	Leu	Glu	Gly	Ser	Leu	Gln	Lys	Arg	Gly	Ile	Val	Glu	Gln	Cys	Cys	85	90	95	
Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn			100	105	110	

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 359 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

195

CCGGGGATCC TTCTGCCATG GCCCTGTGGA TGCGCCTCCT GCCCCTGCTG GCGCTGCTGG 60
 CCCTCTGGGG ACCTGACCCA GCCGCAGCCT TTGTGAACCA ACACCTGTGC GGCTCACACC 120
 TGGTGGAAGC TCTCTACCTA GTGTGCGGGG AACGAGGCTT CTTCTACACA CCAAGACCC 180
 GCCGGGAGGC AGAGGACCTG CAGGTGGGGC AGGTGGAGCT GGGCGGGGGC CCTGGTGCAG 240
 GCAGCCTGCA GCCCTTGGCC CTGGAGGGGT CCCTGCAGAA GCGTGGCATT GTGGAACAAT 300
 GCAGTACTAG CATCTGCTCC CTCTACCAGC TGGAGAACTA CAGCAACTAG ATCTAGCCC 359

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 110 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu
 1 5 10 15
 Trp Gly Pro Asp Pro Ala Ala Ala Phe Val Asn Gln His Leu Cys Gly
 20 25 30
 Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg Gly Phe
 35 40 45
 Phe Tyr Thr Pro Lys Thr Arg Arg Glu Ala Glu Asp Leu Gln Val Gly
 50 55 60
 Gln Val Glu Leu Gly Gly Gly Pro Gly Ala Gly Ser Leu Gln Pro Leu
 65 70 75 80
 Ala Leu Glu Gly Ser Leu Gln Lys Arg Gly Ile Val Glu Gln Cys Ser
 85 90 95
 Thr Ser Ile Cys Ser Leu Tyr Gln Leu Glu Asn Tyr Ser Asn
 100 105 110

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 867 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

196

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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GGATCCATGA ACAGTGAGGA GCAGTACTAC GCGGCCACAC AGCTCTACAA GGACCCGTGC 60
GCATTCCAGA GGGGCCCCGT GCCAGAGTTC AGCGCTAACC CCCCTGCGTG CCTGTACATG 120
GGCCGCCAGC CCCCACCTCC GCCGCCACCC CAGTTTACAA GCTCGCTGGG ATCACTGGAG 180
CAGGGAAGTC CTCCGGACAT CTCCCCATAC GAAGTGCCCC CGCTCGCCTC CGACGACCCG 240
GCTGGCGCTC ACCTCCACCA CCACCTTCCA GCTCAGCTCG GGCTCGCCCA TCCACCTCCC 300
GGACCTTTCC CGAATGGAAC CGAGCCTGGG GGCCTGGAAG AGCCCAACCG CGTCCAGCTC 360
CCTTTCCCGT GGATGAAATC CACCAAAGCT CACGCGTGGA AAGGCCAGTG GGCAGGAGGT 420
GCTTACACAG CGGAACCCGA GGAAAACAAG AGGACCCGTA CTGCCTACAC CCGGGCGCAG 480
CTGCTGGAGC TGGAGAAGGA ATTCTTATTT AACAAATACA TCTCCCGGCC CCGCCGGGTG 540
GAGCTGGCAG TGATGTTGAA CTTGACCGAG AGACACATCA AAATCTGGTT CCAAAACCGT 600
CGCATGAAGT GGAAAAAAGA GGAAGATAAG AAACGTAGTA GCGGGACCCC GAGTGGGGGC 660
GGTGGGGGCG AAGAGCCGGA GCAAGATTGT GCGGTGACCT CGGGCGAGGA GCTGCTGGCA 720
TGCCACCCGC TGCCACCTCC CGGAGGTGCC GTGCCCCCAG GCGTCCCAGC TGCAGTCCGG 780
GAGGGCCTAC TGCCTTCGGG CCTTAGCGTG TCGCCACAGC CCTCCAGCAT CGCGCCACTG 840
CGACCGCAGG AACCCCGGTG AAGATCT

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867

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 284 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```

Met Asn Ser Glu Glu Gln Tyr Tyr Ala Ala Thr Gln Leu Tyr Lys Asp
1           5           10           15
Pro Cys Ala Phe Gln Arg Gly Pro Val Pro Glu Phe Ser Ala Asn Pro
20           25           30
Pro Ala Cys Leu Tyr Met Gly Arg Gln Pro Pro Pro Pro Pro Pro
35           40           45
Gln Phe Thr Ser Ser Leu Gly Ser Leu Glu Gln Gly Ser Pro Pro Asp
50           55           60

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SUBSTITUTE SHEET (RULE 26)

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Ile Ser Pro Tyr Glu Val Pro Pro Leu Ala Ser Asp Asp Pro Ala Gly
 65 70 75 80
 Ala His Leu His His His Leu Pro Ala Gln Leu Gly Leu Ala His Pro
 85 90 95
 Pro Pro Gly Pro Phe Pro Asn Gly Thr Glu Pro Gly Gly Leu Glu Glu
 100 105 110
 Pro Asn Arg Val Gln Leu Pro Phe Pro Trp Met Lys Ser Thr Lys Ala
 115 120 125
 His Ala Trp Lys Gly Gln Trp Ala Gly Gly Ala Tyr Thr Ala Glu Pro
 130 135 140
 Glu Glu Asn Lys Arg Thr Arg Thr Ala Tyr Thr Arg Ala Gln Leu Leu
 145 150 155 160
 Glu Leu Glu Lys Glu Phe Leu Phe Asn Lys Tyr Ile Ser Arg Pro Arg
 165 170 175
 Arg Val Glu Leu Ala Val Met Leu Asn Leu Thr Glu Arg His Ile Lys
 180 185 190
 Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys Glu Glu Asp Lys
 195 200 205
 Lys Arg Ser Ser Gly Thr Pro Ser Gly Gly Gly Gly Glu Glu Pro
 210 215 220
 Glu Gln Asp Cys Ala Val Thr Ser Gly Glu Glu Leu Leu Ala Val Pro
 225 230 235 240
 Pro Leu Pro Pro Pro Gly Gly Ala Val Pro Pro Gly Val Pro Ala Ala
 245 250 255
 Val Arg Glu Gly Leu Leu Pro Ser Gly Leu Ser Val Ser Pro Gln Pro
 260 265 270
 Ser Ser Ile Ala Pro Leu Arg Pro Gln Glu Pro Arg
 275 280

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 677 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGCTTCAGG CTGCCAGCAC ACTATCTGTT ATTGCTGCCA CTGCCCACTG AAAGGGATCT 60

198

TGAGACATGA GGTGCATCTC CAGGCTGCCA GCTGTTCTCC TCATCCTCTC GGTGGCACTC 120
 GGCCACTTGA GAGCTACACC TGTCGGAAGT GGTACCAACC CTCAGGTGGA CAAACGGAAG 180
 TGCAACACAG CCACATGTGC CACACAACGT CTGGCAAAC TCTTGGTTCG CTCCAGCAAC 240
 AACCTTGGTC CAGTCCTCCC ACCAACCAAT GTGGGATCCA ATACATATGG GAAGAGGAAT 300
 GTGGCAGAGG ATCCAAATAG GGAATCCCTG GATTTCCTAC TCCTGTAAAG TCAATGTACT 360
 CCCGTATCTC TTATTACTTC CTGTGTAAAT GCTCTGATGA TTTCCTGAAT AATGTAACAG 420
 TGCCTTCAAC GTGCCTGTGC TTGCTGTGTT TGTAAATTCT TATTCTAAGA CGTGCTTTAA 480
 ACTGAGTGTT GATAAAGGTC AGGGTGAATA CCTCTCTAAT CACAACATGT TCTTGGCTGT 540
 ACATCGATAT CGTAGGAACA CTTAAAATTT CTGTTTTTAC CTTGTAACTC TATGACTCAA 600
 GTTTAACAAT AAAGGAGGGC GTGGGATGGT GGACTTGAAA AGTCATTAAC AGCTCATAGT 660
 AAATTCTCTGA TTCTAGA 677

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met	Arg	Cys	Ile	Ser	Arg	Leu	Pro	Ala	Val	Leu	Leu	Ile	Leu	Ser	Val
1				5					10					15	
Ala	Leu	Gly	His	Leu	Arg	Ala	Thr	Pro	Val	Gly	Ser	Gly	Thr	Asn	Pro
			20				25							30	
Gln	Val	Asp	Lys	Arg	Lys	Cys	Asn	Thr	Ala	Thr	Cys	Ala	Thr	Gln	Arg
		35				40					45				
Leu	Ala	Asn	Phe	Leu	Val	Arg	Ser	Ser	Asn	Asn	Leu	Gly	Pro	Val	Leu
	50					55					60				
Pro	Pro	Thr	Asn	Val	Gly	Ser	Asn	Thr	Tyr	Gly	Lys	Arg	Asn	Val	Ala
65					70					75				80	
Glu	Asp	Pro	Asn	Arg	Glu	Ser	Leu	Asp	Phe	Leu	Leu	Leu			
				85					90						

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2086 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GGATCCAAGG CCCAACTCCC CGAACCACTC AGGTCCTGTG	GGACAGCTCA	CCTAGCTGCA	60
ATGGCTACAG GTAAGCGCCC CTAAATCCC TTGGCACAA	TGTGTCCTGA	GGGGAGGGC	120
AGCGACCTGT AGATGGGACG GGGGCACATA CCCTCAGGT	TTGGGTTTCT	GAATGTAGT	180
ATGGCCATCT AAGCCCAGTA TTGGCCCAT CTCAGAAAGC	TCCTGGCTCC	CTGGAGGATG	240
GAGAGAGAA AACAAACAGC TCCTGGAGCA GGGAGAGTGT	TGGCCTCTTG	CTCTCCGGCT	300
CCCTCTGTG CCCTCTGGTT TCTCCCCAGG CTCCCGGACG	TCCCTGCTCC	TGGCTTTTGG	360
CCTGCTCTGC CTGCCCTGGC TTCAAGAGGG CAGTGCCTTC	CCAACCATTC	CCTTATCCAG	420
GCTTTTGGAC AACGCTATGC TCCGCGGCCA TCGTCTGCAC	CAGCTGGCCT	TTGACACCTA	480
CCAGGAGTTT GTAAGCTCTT GGGGAATGGG TGGGCATCAG	GGGTGGCAGG	AAGGGGTGAC	540
TTTCCCCCGC TGGAAATAAG AGGAGGAGAC TAAGGAGCTC	AGGTTTTC	CCGACCCGGA	600
AAATGAGGC AGATGAGCAC ACGTGTAGCT AGTTTCCCAG	AAAAGTAAAA	TGGAGCAGG	660
TCTCAGCTCA GACCTTGGTG GCGGTCTCTT CTCCTAGGAA	GAAGCTATA	TCCCAAAGGA	720
ACAGAAGTAT TCATTCTCTG AGAACCCCCA GACCTCCCTC	TGTTTCTCAG	AGTCTATTCC	780

GACACCCTCC AACAGGGAGG AAACACAAACA GAAATCCGTG AGTGGATGCC TTCTCCCCCAG 840
GCGGGGATGG GGGAGACCTG TAGTCAGAGC CCCCGBGCAG CACAGCCAAT GCCCGTCCCTT 900
GCCCCGTCAG AACCTAGAGC TGCTCCGCAT CTCCTGCTG CTCATCCAGT CGTGGGTGGA 960
GCCCCGTGCAG TTCCTCAGGA GTGTCTTCGC CAACAGCCTG GTGTACGGCG CCTCTGACAG 1020
CAACGTCTAT GACCTCCTAA AGGACCTAGA GGAAGGCATC CAAACGCTGA TGGGGGTGAG 1080
GGTGGCGCCA GGGGTCCCCA ATCCTGGAGC CCACTGACT TTGAGAGACT GTGTTAGAGA 1140
AACACTGGCT GCCCTCTTTT TAGCAGTCAG GCCCTGACCC AAGAGAACTC ACCTTATTCT 1200
TCATTTCCCC TCGTGAATCC TCCAGGCCTT TCTCTACACT GAAGGGGAGG GAGGAAATG 1260
AATGAATGAG AAAGGGAGGG AACAGTACCC AAGCGCTTGG CCTCTCCTTC TCTTCCCTCA 1320
CTTTGCAGAG GCTGGAAGAT GGCAGCCCCC GGAAGTGGCA GATCTTCAAG CAGACCTACA 1380
GCAAGTTCGA CACAAACTCA CACAACGATG ACGCACTACT CAAGAAGTAC GGGCTGCTCT 1440
ACTGCTTCAG GAAGGACATG GACAAGGTCG AGACATTCTT GCGCATCGTG CAGTGCCGCT 1500
CTGTGGAGGG CAGCTGTGGC TTCTAGCTGC CCGGTGGCA TCCCTGTGAC CCCTCCCCAG 1560
TGCCCTCTCT GGCCTGGAA GTTGCCACTC CAGTGCCAC CAGCCTTGTC CTAATAAAAT 1620
TAAGTTGCAT CATTTGTCT GACTAGGTGT CCTTCTATAA TATTATGGGG TGGAGGGGGG 1680
TGGTATGGAG CAAGGGGCC AAGTTGGAA GACAACCTGT AGGGCCTGCG GGGTCTATTTC 1740
GGGAACCAAG CTGGAGTGCA GTGGCACAAAT CTGGGCTCAC TGCAATCTCC GCCTCCTGGG 1800

TTCRAGCGAT TCTCTGCCT CAGCCTCCG AGTTGTTGGG ATTCCAGGCA TGCATGACCA 1860
 GGCTCAGCTA ATTTTGTGTT TTTTGGTAGA GACGGGGTTT CACCATAATTG GCCAGGCTGG 1920
 TCTCCAACTC CTAATCTCAG GTGATCTACC CACCTTGGCC TCCCAAATTG CTGGGATTAC 1980
 AGGCGTGAAC CACTGCTCCC TTCCCTGTCC TTCTGATTTT AAAATAACTA TACCAGCAGG 2040
 AGGACGTCCA GACACAGCAT AGGTACCTG CCATGGCCCA ACCGGT 2086

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 217 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
 1 5 10 15
 Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
 20 25 30
 Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
 35 40 45
 Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
 50 55 60
 Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
 65 70 75 80

202

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Thr Gln Gln Lys
 85 90 95
 Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Ile Gln Ser Trp
 100 105 110
 Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
 115 120 125
 Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
 130 135 140
 Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
 145 150 155 160
 Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
 165 170 175
 His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
 180 185 190
 Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
 195 200 205
 Arg Ser Val Glu Gly Ser Cys Gly Phe
 210 215

(2) INFORMATION FOR SEQ ID NO: 11:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 235 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGGATCCAG GTCGACGCCG GCCAAGACAG CACAGACAGA TTGACCTATT GGGGTGTTTC 60
GCGAGTGTGA GAGGGAAGCG CCGGGCCTG TATTCTAGA CCTGCCCTTC GCCTGGTTCC 120
TGGCGCCTTG TGACCCCGGG CCCCTGCCGC CTGCAAGTCG AAATTGCGCT GTGCTCCTGT 180
GCTACGGCCT GTGGCTGGAC TGCCTGCTGC TGCCCAACTG GCTGGCAAGA TCTCG 235

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 441 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGTACCGGCG CCCCCCTCGA GGTCTTGAGC TAAGRATCCA GCTATCAATA GAACTATGA 60
AACAGTTCCA GGGACAAAGA TACCAGGTCC CCAACAACTG CAACTTTCTG GGAATGAGG 120
TGGAAATGC TCAGCCAAGG AAAAAGAGGG CCTTACCCTC TCTGGGACAA TGATTGTGCT 180
GTGAACTGCT TCATCAGGC ATCTGGCCCC TTGTTAATAA TCTAATTACC CTAGGTCTAA 240
GTAGAGTTGT TGACGTCCAA TGAGCGCTTT CTGCAGACTT AGCACTAGGC AAGTGTTTGG 300
AAATTACAGC TTCAGCCCCT CTCGCCATCT GCCTACCTAC CCCTCCTAGA GCCCTTAATG 360

GGCCHAACGG CAAAGTCCAG GGGSCAGAGA GGAGGTGCTT TGGACTATAA AGCTAGTGGA 420
GACCCAGTAA CTCCAAGCT T 441

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1252 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GGGTATCTTT GCCCGATGG ATGCTGGGCC TAGCCAGGG CTCCCAGTCC CCAGGCGTGG 60
GGTAGAAGTT GGA CTCTATA GTCACCTAAG GGCCTATGTT GCAGTCCTGG TCTCAGGCAC 120
GCGGCTGCA GGAGAGGCTT AAAAGAAGAG AAACCTGCACA CAATGGCTAG GTCACCGGCG 180
TTAAAGCTAA GCAAAACCCAG CGTACTCTCT GGGCAGCAAC TGCAAAAGCGT TTCTTCAGGT 240
CCCTCACCTG TAGAATCAGA GCGGTAGTCG CCTCTGCATG TCTGAGTTCT TACATGTCGT 300
AATGTACAA CACGATTTC CCTCAATCAC CGCCCGGAAC AGTACCTCCA ACTTCCCAGA 360
CCCGGATGCC CCAAGAGCCA GAGTAGGGTG GGAATAATCGG GACAGGCCCC CAAATTCCAC 420
TCGGGGGCTT TGAGCTCTTA CATGGTGTCA CGGGGGCAGG TAGTTTGGGT TTAGCAATGT 480
GAACCTGAC AATTGGGAT GTAGAGCTGG TGGGCCATCG TGGGACGCCA AGCATCATCC 540
TTAGAGTTTG GATCCTTTAG GGCAGGCAGG CACAGGGACC CAGTGCAGAG TCAGTGAAGC 600

CGCCAGTTT CGGCTTCCG TCCTTTTCCA CCCCCACTTG CGTGCTTCTC CAACAGTGTG 660
 GATGGAGGG GTGGGGGACG AGCCCTAATC TCCGAGGAAG GGGTGTGGCC CCGTTCGTGT 720
 TCTCCAGTTT GTGGCGTCCT GGATCTGTCC TCTGGTCCCC TCCAGATCGT GTCCCAACACC 780
 CACCCGTTCA GGCATGGCAC TGTGCCGCCA CGCGTGACCG TGCCTCTCTT ACGTGGGGGA 840
 CGTGCAGGGT GTGCTCTCCT TTCCGGTGGC GGAGGGAGCG GCCGTCTTTC TCCTGCTCTG 900
 GCTGGGAAGC CCCAGCCATT GCGCTGCAGA GGAGACTTGC AGCCAATGGG GACTGAGGAA 960
 GTGGGCCGGC TGGCGGTTGT CACCCTCCCG GGGACCGGAG CTCCGAGGTC TGGAGAGCGC 1020
 AGCAGACGC CCGCCCCGCC CGGGGACTGA GGGGGAGGAG CGAAGGGAGG AGGAGGTGGA 1080
 GTCTCCGATC TGCCGCTGGA GGACCACTGC TCACCAGGCT ACTGAGGAGC CACTGGCCCC 1140
 ACACCTGCTT TTCCGCATCC CCCACCGTCA GCATGATCGC CGCGCAACTA CTGGCCTATT 1200
 ACTCACCGAG CTGAAGGATG ACCAAGTCAA AAAGGTGAGC CCGCGCGGCG CC 1252

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 410 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAATTCTGTT GGGCTCGCGG TTGACCACAA ACTCTTCGG GTCTTCCAG TACTCTTGGA 60

TCGGAACCC GTCGGCCTCC GAACGGTACT CCGCCACCGA GGGACCTGAG CGAGTCCGCA 120
 TCGACCGGAT CGGAACCT CTCGACTGTT GGGGTGAGTA CTCCTCTCTCA AAAGCGGGCA 180
 TGACTTCTGC GCTAAGATTG TCAGTTTCCA AAAACGAGGA GGATTTGATA TTCACCTGGC 240
 CCGCGGTGAT GCCTTTGAGG GTGGCCGCGT CCATCTGGTC AGAAAAGACA ATCTTTTGTG 300
 TGTCAGCTT GAGGTGTGGC AGGCTTGAGA TCTGGCCATA CACTTGAGTG ACAATGACAT 360
 CCACTTGGC TTTCTCTCAC ACAGGTGTCC ACTCCAGGT CCAACTGCAG 410

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GATCCCTCA TCAGGCCATC TGGCCCTTG TTAATAATCG ACTGACCCTA GGTCTAAGAT 60
 CCCTTCATCA GGCCATCTGG CCCCTTGTTA ATAATCGACT GACCCTAGGT CTAAGATCCC 120
 TTTCATCAGGC CATCTGGCCC CTGTGTTAATA ATCGACTGAC CCTAGGTCTA AGATC 175

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTCCCTCG AGCACCGCC GGAACAGTAC C

31

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTTGGCCTC GAGCATGCTG ACGTGGGGG

30

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GTTGGACTCG AGAGTCACCT AAGGGCCTAT G

31

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GATTGGGAAG ACAATAGCAG GCATGC

26

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGTCGCCTCT GCATGTCTGA GTTC

24

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

209

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CTTGAGCTCT TACATGGTGT CACG

24

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

TCCCCAGCG TGGGTAGAA G

21

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CAACCGGTGG GACATTGAG TTGC

24

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs

210

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CCAAGTCATT ATAGATCAT AGTC

24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCGGATCCCA TGATTGAACA AGAT

24

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CCAAGATCTC GCTCAGAAGA ACTC

24

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCGATCCAG GTCGAGCCG GCCAA

25

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CGAGATCTTG CCAGCCAGTT GG

22

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GATCCCTTCA TCAGGCCATC TGGCCCTTG TTAATAATCG ACTGACCCTA GGTCTAA

57

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GATCTTAGAC CTAGGGTCAG TCGATTATTA ACAAGGGGCC AGATGGCCTG ATGAAGG

57

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CTCCCAAGCT TAAGTGACCA GCTACAA

27

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs

213

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GGGCAACCTA GGTACTGGAC CTTCTATC

28

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GGGTCTAGAG GACCTGTTCC CACCG

25

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GCCGATTCG AGGAGCAGAG AGCGRAGC

28

214

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

GCCAGCAGGG GCAGGAGGCG CATCCACAGG GCCAT

35

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CCACCTGTCT ACACCTCCTC TC

22

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GTAATCCAGG TGTCGTGACT GC

22

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CCGGGGATCC TTCTGCCATG GCCC

24

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GGGCTAGATC TAGTTGCTGT AGTTCTCCAG CTGGTAGAGG GAGCAGATGC TAGTACTGCA

60

TTGTTCCAC

69

(2) INFORMATION FOR SEQ ID NO: 40:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

GGGCTAGATC TAGTGCAGT AGTTCTC

27

(2) INFORMATION FOR SEQ ID NO: 41:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

GGATCCATGA ACAGTGAGGA GCAG

24

(2) INFORMATION FOR SEQ ID NO: 42:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

AGATCTTAC CGGGTTCCT GCGG

24

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

GGGGATCCGA TATGAAAAAG CCTG

24

(2) INFORMATION FOR SEQ ID NO: 44:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

CGAGATCTAC TCTATTCCTT TGC

23

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs

218

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

CCGGATCCCA TGAGCGAAAA AT

22

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

GGAGATCTTT AGCGACCGGA GAT

23

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

CCGGATCCAT GGTTCGACCA TTG

23

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

GGAGATCTGT TAGTCTTTCT TC

22

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

CCGGATCCAT GAGCTTCAAT AC

22

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

23

CCAGATCTGC TCATGCTTGC TCC

(2) INFORMATION FOR SEQ ID NO: 51:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 68 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

60

GCTATTTCGGC TAGGACTGGG CACAATTITU UGUGCCCACT CCTAGCCGAA UAGCGGCGGT

68

TTTCGCGC

(2) INFORMATION FOR SEQ ID NO: 52:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 69 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc_RNA
- (B) LOCATION: 30..39

(ix) FEATURE:

- (A) NAME/KEY: misc_RNA
- (B) LOCATION: 45..55

221

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TCACCGGAAC CTAGGCTTC ACTGTTTTC CAGUGAAAGC CTAGGUUCCG GUUGAGCGCG 60

TTTTCGCGC 69

(2) INFORMATION FOR SEQ ID NO: 53:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 91 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION:1..2
- (D) OTHER INFORMATION:/note= "Phosphorothioate linkage
between bases 1 and 2"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION:90..91
- (D) OTHER INFORMATION:/note= "Phosphorothioate linkage
between bases 90 and 91"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GGTTCCTCC AGTTCGGATA TGACATCGGT GTGATCAATG CACCTTAAGA GGTAATAATA 60

TCCCATATC GACATGTTTT GGGTGTTCCT C 91

222

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 91 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..2
- (D) OTHER INFORMATION: /note= "Phosphorothioate linkage
between bases 1 and 2"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 90..91
- (D) OTHER INFORMATION: /note= "Phosphorothioate linkage
between bases 90 and 91"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

GAGGAACACC CAAACATGT CGATAATGGG ATATTATTAC CTCTTAAGGT GCATTGATCA 60
CACCGATGTC ATATCCGAAC TGAAGGAAC C 91

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

223

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION:1..2
 (D) OTHER INFORMATION:/note= "Phosphorothioate linkage
 between bases 1 and 2"

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION:60..61
 (D) OTHER INFORMATION:/note= "Phosphorothioate linkage
 between bases 60 and 61"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

GGATATGACA TCGGTGTGAT CAATGCACCT TAAGAGGTAA TAATATCCCA TTATCGACAT 60

G 61

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 61 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION:1..2
 (D) OTHER INFORMATION:/note= "Phosphorothioate linkage
 between bases 1 and 2"

(ix) FEATURE:
 (A) NAME/KEY: misc_feature

224

(B) LOCATION: 60..61

(D) OTHER INFORMATION: /note= "Phosphorothioate linkage
between bases 60 and 61"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

CATGTCGATA ATGGGATATT ATTACCTCTT AAGGTGCATT GATCACACCG ATGTCATATC

60

C

61

SUBSTITUTE SHEET (RULE 26)

CLAIMS

1. A method for the production of a polypeptide comprising the steps of:

- 5 (a) providing a secretory host cell;
- (b) blocking the production of an endogenous, secreted polypeptide;
- (c) contacting with said host cell an exogenous polynucleotide comprising a gene encoding an exogenous polypeptide, wherein said gene is under the control of a promoter active in eukaryotic
- 10 cells; and
- (d) culturing said secretory host cell under conditions such that said exogenous polynucleotide expresses said exogenous polypeptide.

15 2. The method of claim 1, wherein said promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1.

20 3. The method of claim 1, wherein said exogenous polynucleotide further comprises an adenovirus tripartite 5' leader sequence and intron.

25 4. The method of claim 3, wherein said intron comprises the 5' donor site of the adenovirus major late transcript and the 3' splice site of an immunoglobulin gene.

5. The method of claim 1, wherein said exogenous polynucleotide further comprises a polyadenylation signal.

30

6. The method of claim 1, wherein said secretory host cell is a neuroendocrine cell.

5 7. The method of claim 6, wherein said secretory host cell is an insulinoma cell.

10 8. The method of claim 7, wherein said insulinoma cell is a rat insulinoma cell.

9. The method of claim 7, wherein said insulinoma cell is a human insulinoma cell.

15 10. The method of claim 1, wherein said exogenous polypeptide is secreted.

20 11. The method of claim 10, wherein said exogenous polypeptide is fusion protein.

25 12. The method of claim 11, wherein said fusion protein comprises a leader sequence that is not naturally associated with said exogenous polypeptide.

13. The method of claim 10, wherein said exogenous polypeptide is amidated.

14. The method of claim 13, wherein said amidated polypeptide is selected from the group consisting of calcitonin, calcitonin gene related peptide (CGRP), b-calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40) (PTH-rP), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), cholecystokinin (27-33) (CCK), galanin message associated peptide, preprogalanin (65-105), gastrin I, gastrin releasing peptide, glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalins, enkephalinamide, metorphinamide (adrenorphin), alpha melanocyte stimulating hormone (alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

15

15. The method of claim 10, wherein said exogenous polypeptide is a hormone.

20

16. The method of claim 15, wherein said hormone is selected from the group consisting of growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I, angiotensin II, b-endorphin, b-melanocyte stimulating hormone (b-MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins and somatostatin.

25

17. The method of claim 10, wherein said exogenous polypeptide is a growth factor.

30

18. The method of claim 17, wherein said growth factor is selected from the group consisting of epidermal growth factor, platelet-derived growth factor, fibroblast growth factor, hepatocyte growth factor and insulin-like growth factor 1.

5

19. The method of claim 1, wherein said endogenous, secreted polypeptide and said exogenous polypeptide are the same.

10

20. The method of claim 19, wherein said endogenous, secreted polypeptide and said exogenous polypeptide are insulin.

15

21. The method of claim 1, wherein said exogenous polypeptide enhances the production and/or secretion of at least one polypeptide produced by said cell.

20

22. The method of claim 21, wherein said exogenous polypeptides is selected from the group consisting of a protein processing enzyme, a receptor and a transcription factor.

25

23. The method of claim 22, wherein said exogenous polypeptide is selected from the group consisting of hexokinase, glucokinase, GLUT-2, GLP-1, IPF1, PC2, PC3, PAM, glucagon-like peptide I receptor, glucose-dependent insulinotropic polypeptide receptor, BIR, SUR, GHRFR and GHRHR.

24. The method of claim 1, wherein said step (c) further comprises contacting said secretory host cell with a polynucleotide comprising a gene for a selectable marker and step (d) further comprises culturing under drug selection.

5

25. The method of claim 24, wherein said selectable marker gene is flanked by *LoxP* sites.

10

26. The method of claim 25, further comprising:

(e) contacting the secretory host cell with a polynucleotide encoding the *Cre* protein, wherein said polynucleotide is under the control of a promoter active in eukaryotic cells; and

15

(f) replicate culturing said cell with and without drug selection.

27. The method of claim 24, wherein said selectable marker is hygromycin resistance and said drug is hygromycin.

20

28. The method of claim 24, wherein said selectable marker is neomycin and said drug is G418.

25

29. The method of claim 24, wherein said selectable marker is GLUT-2 and said drug is streptozotocin.

30. The method of claim 24, wherein the genes for said exogenous polypeptide and said selectable marker are part of the same polynucleotide.

5 31. The method of claim 30, wherein the genes for said exogenous polypeptide and said selectable marker are separated on the same polynucleotide by an internal ribosome entry site.

10 32. The method of claim 1, wherein said secretory host cell is glucose-responsive.

15 33. The method of claim 1, wherein said secretory host cell is not glucose-responsive.

20 34. The method of claim 1, wherein said blocking of production of said endogenous, secreted polypeptide is effected by expression of an RNA antisense to the DNA or mRNA of said endogenous, secreted polypeptide.

25 35. The method of claim 1, wherein said blocking of production of said endogenous, secreted polypeptide is effected by expression of a ribozyme specific for the mRNA of said endogenous, secreted polypeptide.

30 36. The method of claim 1, wherein said blocking of production of said endogenous, secreted polypeptide is effected by interruption of the gene encoding said endogenous, secreted polypeptide.

37. The method of claim 36, wherein said interruption is effected by homologous recombination.

5 38. The method of claim 36, wherein said interruption is effected by genomic site-directed mutagenesis.

10 39. The method of claim 36, wherein said interruption is effected by random integration.

15 40. A secretory host cell comprising an exogenous polynucleotide comprising a gene encoding a first exogenous polypeptide, wherein said secretory host cell is blocked in the expression of at least one endogenous, secreted polypeptide.

20 41. The secretory host cell of claim 40, wherein said exogenous polynucleotide is inserted into the coding or regulatory region of said endogenous, secreted polypeptide.

25 42. The secretory host cell of claim 41, wherein said exogenous polynucleotide further comprises a promoter active in eukaryotic cells.

43. The secretory host cell of claim 42, wherein said promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1.

44. The secretory host cell of claim 40, wherein said exogenous polynucleotide further comprises an adenovirus tripartite 5' leader sequence and intron.

5 45. The secretory host cell of claim 44, wherein said intron comprises the 5' donor site of the adenovirus major late transcript and the 3' splice site of an immunoglobulin gene.

10 46. The secretory host cell of claim 42, wherein said exogenous polynucleotide further comprises a polyadenylation signal.

15 47. The secretory host cell of claim 42, wherein said exogenous polynucleotide comprises a gene encoding a second exogenous polypeptide.

20 48. The secretory host cell of claim 47, wherein the genes encoding said first and said second exogenous polypeptides are separated by an internal ribosome entry site.

25 49. The secretory host cell of claim 48, wherein said second exogenous polypeptide is a selectable marker.

50. The secretory host cell of claim 49, wherein said selectable marker is hygromycin resistance.

51. The secretory host cell of claim 49, wherein said selectable marker is neomycin.

5 52. The secretory host cell of claim 49, wherein said selectable marker is GLUT-2.

10 53. A method of propagating the secretory host cell of claim 40, comprising stirring a suspension of said secretory host cell.

15 54. A method of propagating the secretory host cell of claim 40, comprising gas stream agitation of a suspension of said secretory host cell.

20 55. A method of propagating the secretory host cell of claim 40, comprising incubation of said secretory host cell in a non-perfused attached cell container or a perfused attached cell container.

25 56. A method of propagating the secretory host cell of claim 40, comprising culture on microcarriers.

57. A method of propagating the secretory host cell of claim 40, comprising microencapsulation of said secretory host cell, followed by cell culture.

58. A method of propagating the secretory host cell of claim 40, comprising incubation of said secretory host cell in a perfused packed bed reactor.

5 59. The method of claim 58, wherein said incubation is in serum free medium.

60. The method of claim 58, wherein said incubation is in medium supplemented with cholesterol rich lipid extract at a final concentration of 0.01% to 0.10%, volume to volume.

10

61. The method of claim 58, wherein said incubation is in medium supplemented with ascorbic acid at a final concentration of between about 0.001 to 0.100 mM.

15

62. The method of claim 58, wherein said incubation is in medium lacking glutamine and supplemented with glutamate at a final concentration of between about 2 to 20 mM.

20

63. The method of claim 58, wherein said incubation is in medium lacking glutamine and supplemented with alpha-ketoglutarate at a final concentration of between about 2 to 20 mM.

25

64. A recombinant host cell having a human insulin content per 1×10^6 cells of at least about 1000 ng.

30

65. The recombinant host cell of claim 64, having a human insulin content per 1×10^6 cells of at least about 1250 ng.

5 66. The recombinant host cell of claim 65, having a human insulin content per 1×10^6 cells of at least about 1500 ng.

10 67. The recombinant host cell of claim 66, having a human insulin content per 1×10^6 cells of about 2500 ng.

15 68. A recombinant host cell that secretes at least about 200 ng of human insulin per 1×10^6 cells per hour.

 69. The recombinant host cell of claim 68, secreting at least about 300 ng of human insulin per 1×10^6 cells per hour.

20 70. The recombinant host cell of claim 69, secreting at least about 400 ng of human insulin per 1×10^6 cells per hour.

25 71. The recombinant host cell of claim 70, secreting at least about 500 ng of human insulin per 1×10^6 cells per hour.

30 72. The recombinant host cell of claim 71, secreting about 1000 ng of human insulin per 1×10^6 cells per hour.

73. A recombinant host cell secreting at least about 25 mg of human growth hormone per 1×10^6 cells per hour.

5 74. The recombinant host cell of claim 73, secreting at least about 50 mg of human growth hormone per 1×10^6 cells per hour.

10 75. The recombinant host cell of claim 74, secreting about 200 mg of human growth hormone per 1×10^6 cells per hour.

76. A method of preventing type I diabetes comprising the steps of:

- 15 (a) identifying a subject at risk of type I diabetes; and
 (b) providing to said subject a polynucleotide comprising a gene for a disulfide mutant of human insulin, wherein said mutant gene is under the control of a promoter active in eukaryotic cells.

20 77. The method of claim 76, wherein said promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1.

25 78. The method of claim 76, wherein said polynucleotide further comprises an adenovirus tripartite 5' leader sequence and intron.

30 79. The method of claim 78, wherein said intron comprises the 5' donor site of the adenovirus major late transcript and the 3' splice site of an immunoglobulin gene.

80. The method of claim 76, wherein said polynucleotide further comprises a polyadenylation signal.

5 81. The method of claim 76, wherein said providing comprises introducing said polynucleotide to a cell of said subject *in vivo*.

10 82. The method of claim 76, wherein said providing comprises contacting with a secretory host cell *ex vivo* and administering said secretory host cell to said subject.

15 83. The method of claim 82, wherein said secretory host cell is a neuroendocrine cell.

20 84. The method of claim 83, wherein said neuroendocrine cell is an insulinoma cell.

25 85. The method of claim 84, wherein said insulinoma cell is a rat insulinoma cell.

30 86. The method of claim 85, wherein said insulinoma cell is a human insulinoma cell.

87. The method of claim 82, wherein expression of the endogenous insulin in said secretory host cell has been blocked.

88. The method of claim 87, wherein said blocking of production of said endogenous insulin is effected by expression of an RNA antisense to the DNA or mRNA of said endogenous insulin gene.

5

89. The method of claim 87, wherein said blocking of production of said endogenous insulin is effected by expression of a ribozyme specific for the mRNA of said endogenous insulin gene.

10

90. The method of claim 87, wherein said blocking of production of said endogenous insulin is effected by interruption of the gene encoding said endogenous insulin.

15

91. The method of claim 90, wherein said interruption is effected by homologous recombination.

20

92. The method of claim 90, wherein said interruption is effected by genomic site-directed mutagenesis.

25

93. The method of claim 90, wherein said interruption is effected by random integration.

30

94. The method of claim 82, wherein said cell is glucose-responsive.

95. The method of claim 76, wherein said polynucleotide is contained within a packageable, replication defective adenoviral expression construct.

5 96. A method for treating a subject afflicted with diabetes comprising the steps of:

- (a) identifying a subject afflicted with diabetes; and
- (b) providing to said subject a secretory host cell, wherein (i)
10 the production of an endogenous, secreted polypeptide has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding insulin, wherein said gene is under the control of a promoter active in eukaryotic cells.

15 97. The method of claim 96, wherein said promoter is selected from the group consisting of CMV, SV40 IE, RSV LTR, GAPHD and RIP1.

20 98. The method of claim 96, wherein said polynucleotide further comprises an adenovirus tripartite 5' leader sequence and intron.

25 99. The method of claim 98, wherein said intron comprises the 5' donor site of the adenovirus major late transcript and the 3' splice site of an immunoglobulin gene.

30 100. The method of claim 96, wherein said polynucleotide further comprises a polyadenylation signal.

101. The method of claim 96, wherein said secretory host cell is a neuroendocrine cell.

5 102. The method of claim 101, wherein said neuroendocrine cell is an insulinoma cell.

10 103. The method of claim 102, wherein said insulinoma cell is a rat insulinoma cell.

104. The method of claim 102, wherein said insulinoma cell is a human insulinoma cell.

15 105. The method of claim 96, wherein said blocking of production of said endogenous polypeptide is effected by expression of an RNA antisense to the DNA or mRNA of said endogenous polypeptide.

20 106. The method of claim 96, wherein said blocking of production of said endogenous polypeptide is effected by expression of a ribozyme specific for the mRNA of said endogenous polypeptide.

25 107. The method of claim 96, wherein said blocking of production of said endogenous polypeptide is effected by interruption of the gene encoding said endogenous polypeptide.

108. The method of claim 96, wherein said interruption is effected by homologous recombination.

5 109. The method of claim 96, wherein said interruption is effected by genomic site-directed mutagenesis.

10 110. The method of claim 96, wherein said interruption is effected by random integration.

111. The method of claim 96, wherein said cell is glucose-responsive.

15 112. A method for providing a polypeptide to an animal comprising the step of providing to an animal a secretory host cell, wherein (i) the production of an endogenous, secreted polypeptide in said secretory host cell has been blocked and (ii) wherein the secretory host cell comprises an exogenous polynucleotide comprising a gene encoding
20 said polypeptide wherein said gene is under the control of a promoter active in eukaryotic cells.

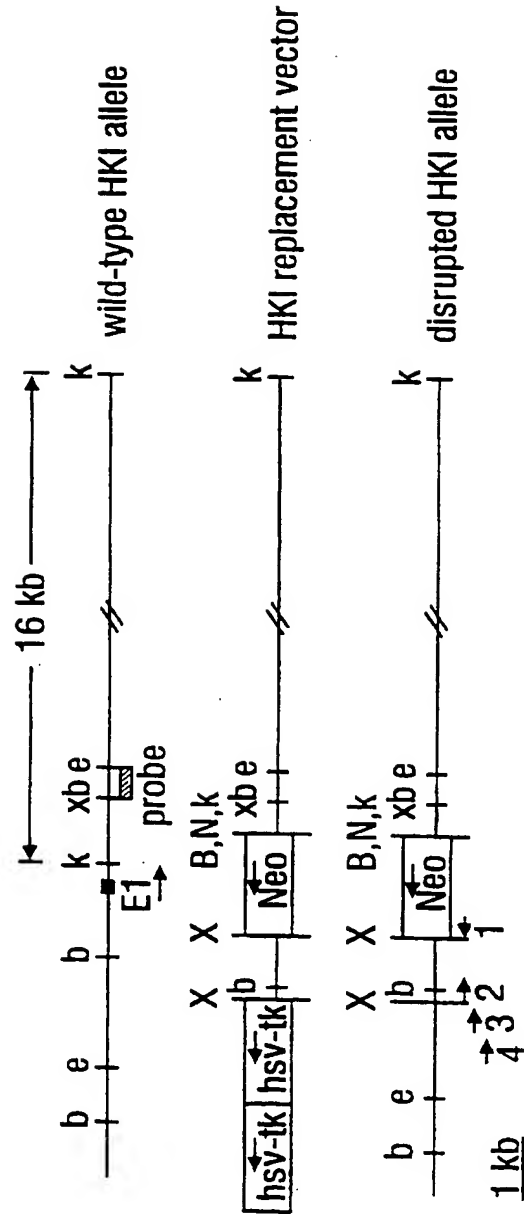


FIG. 1

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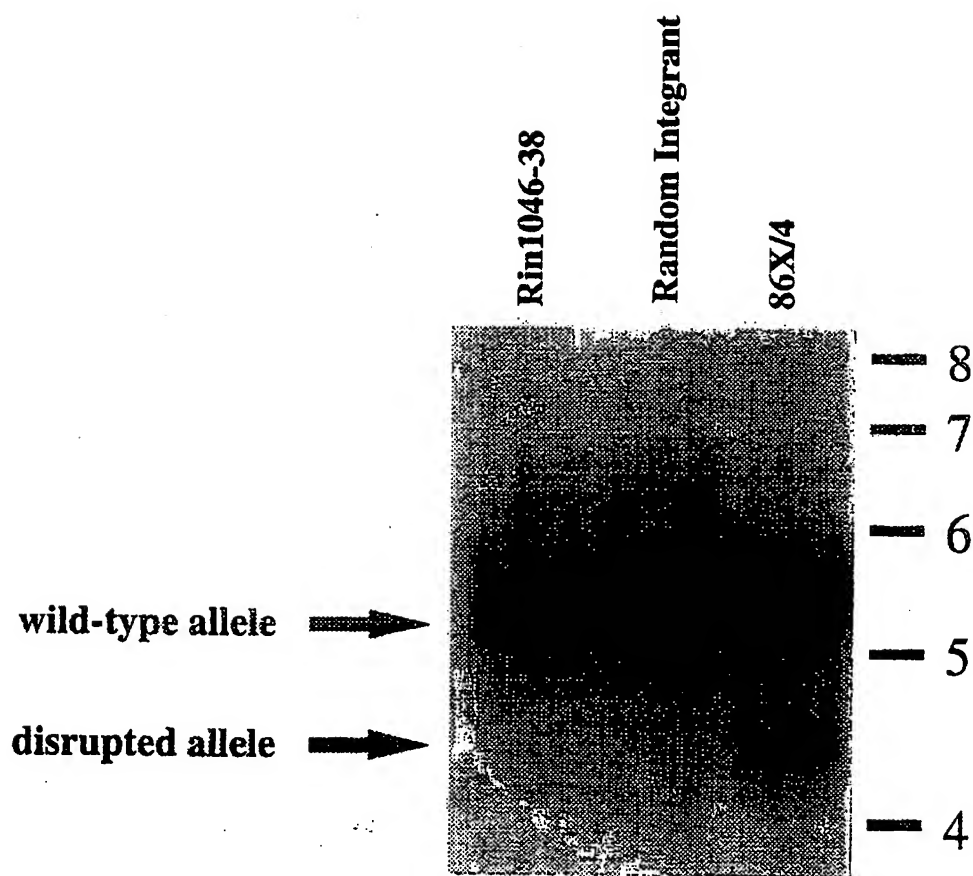


FIG. 2

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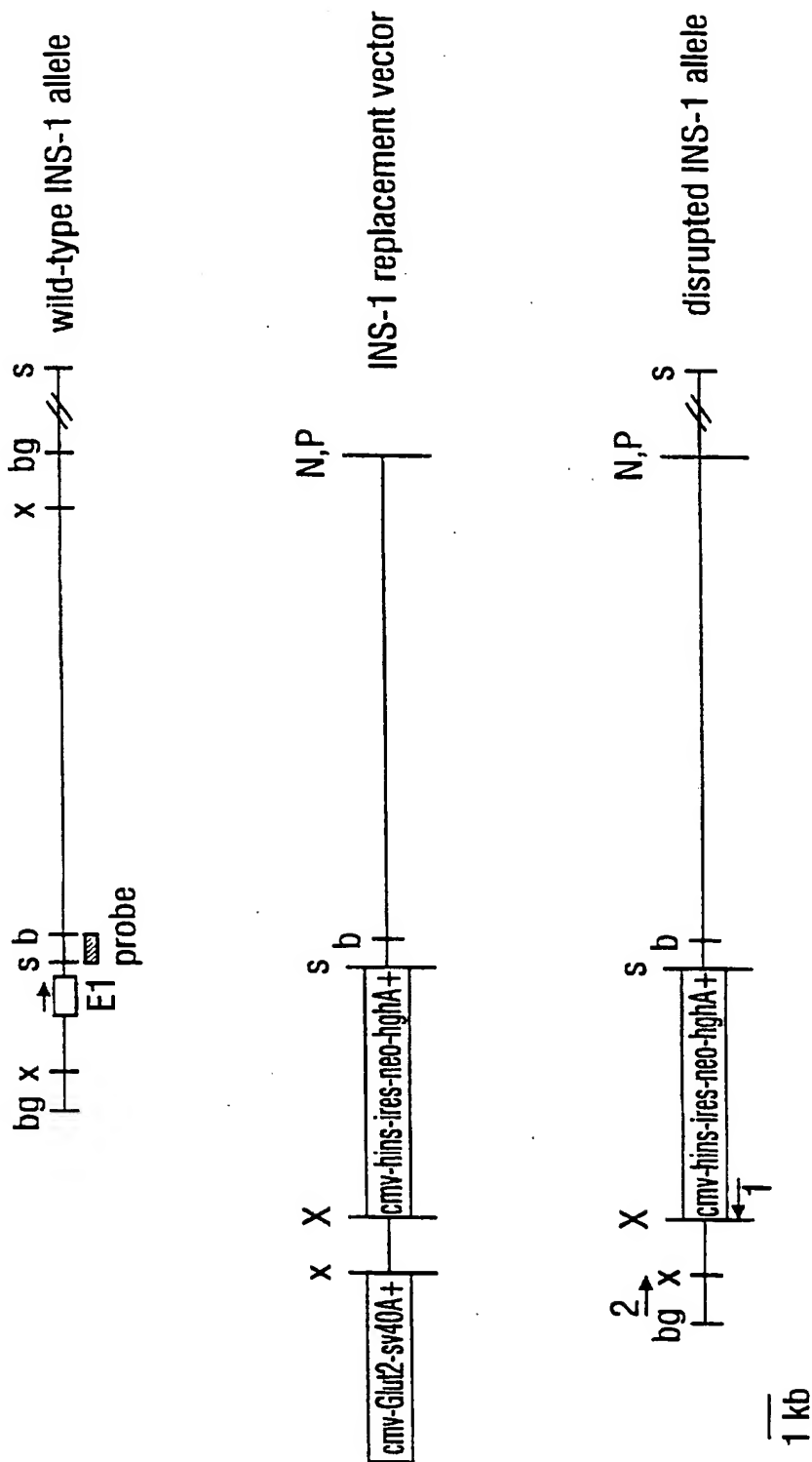


FIG. 3

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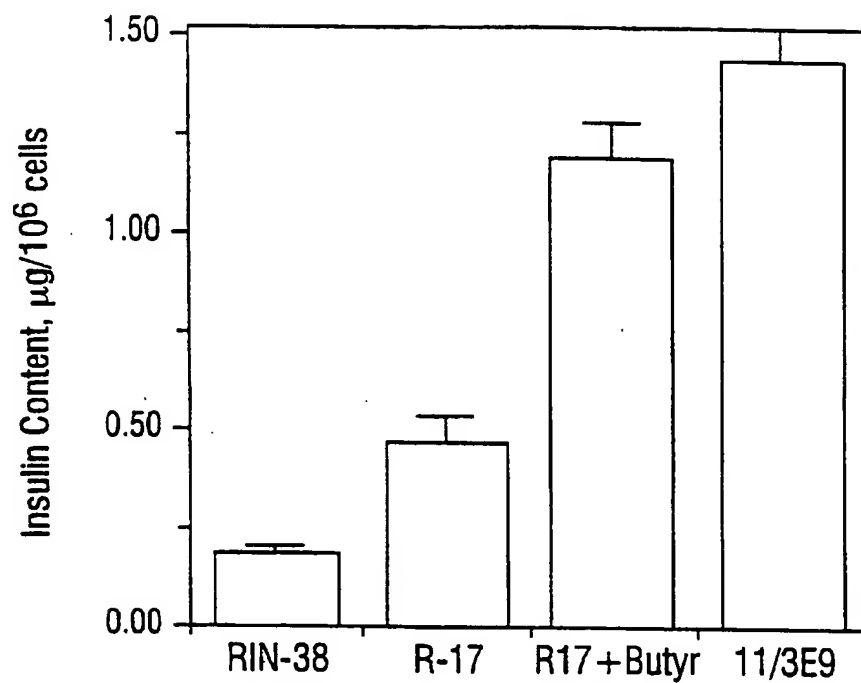


FIG. 4A

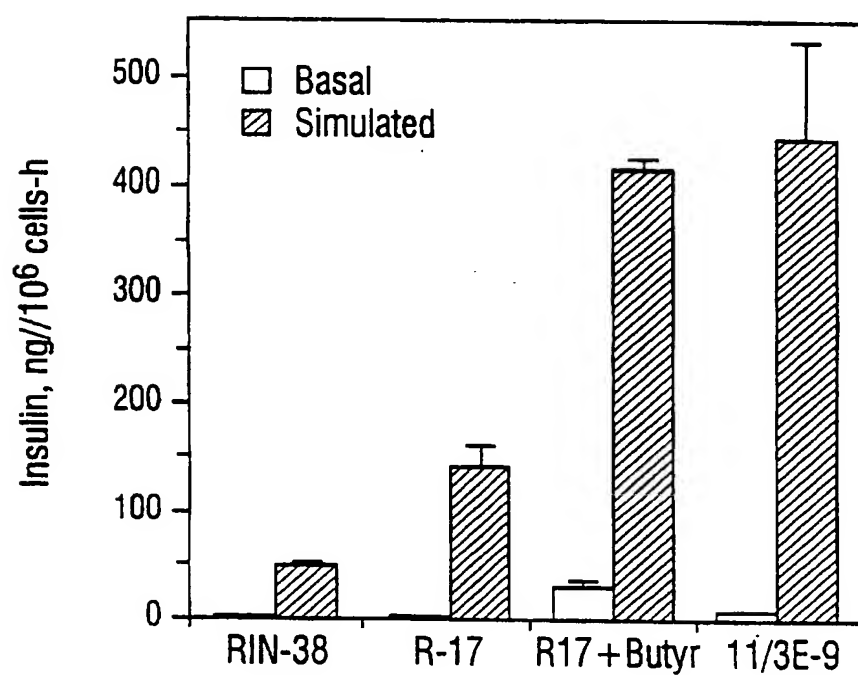


FIG. 4B

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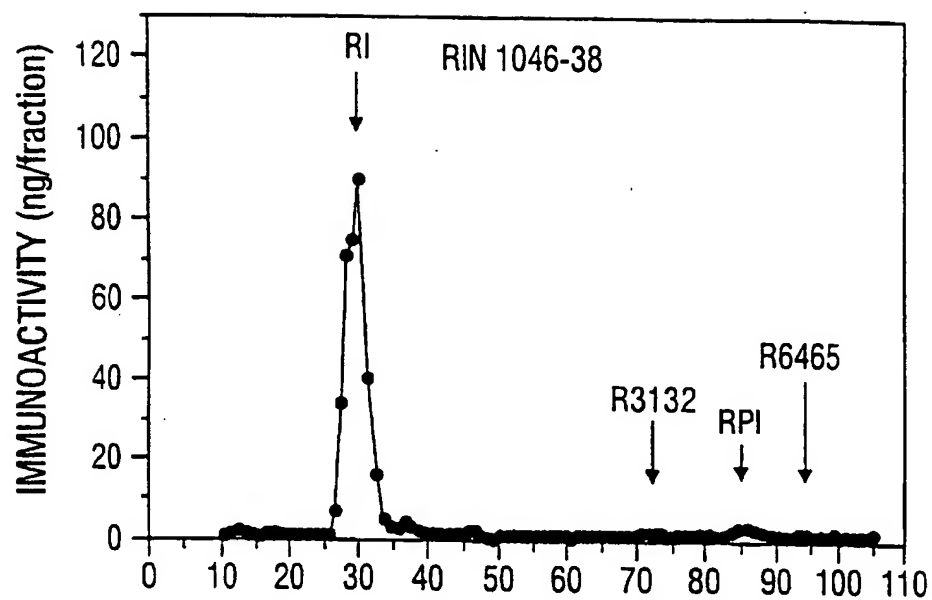


FIG. 5A

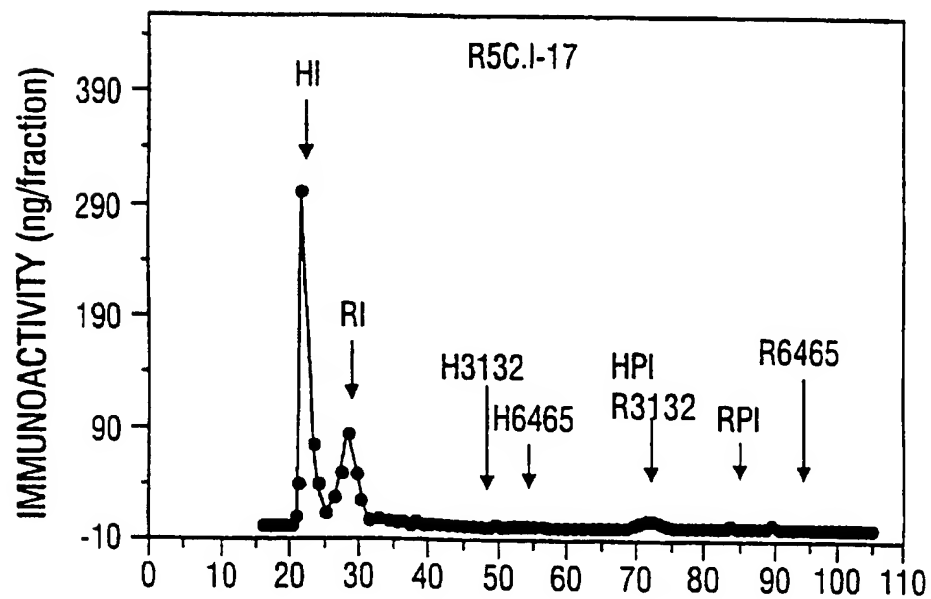


FIG. 5B

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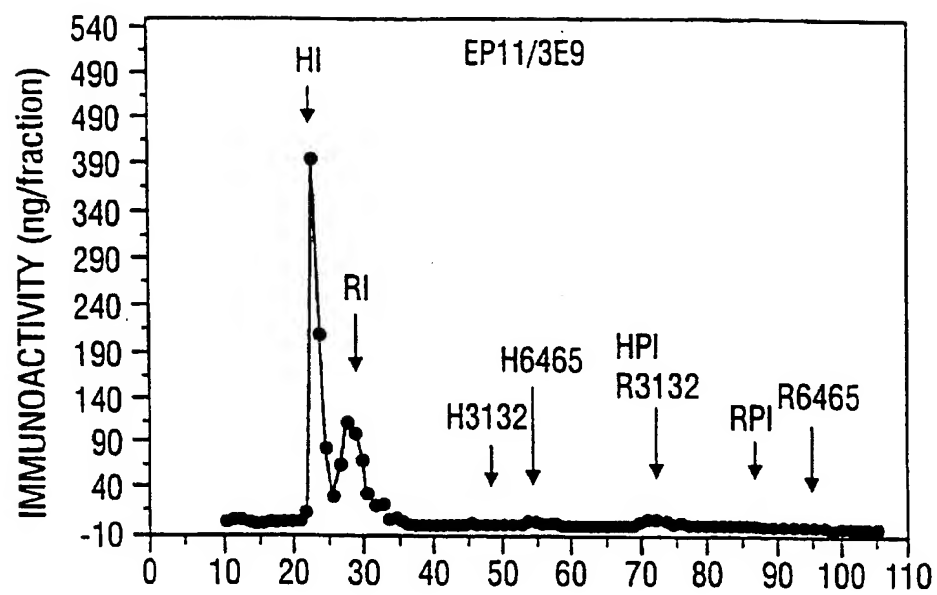


FIG. 5C

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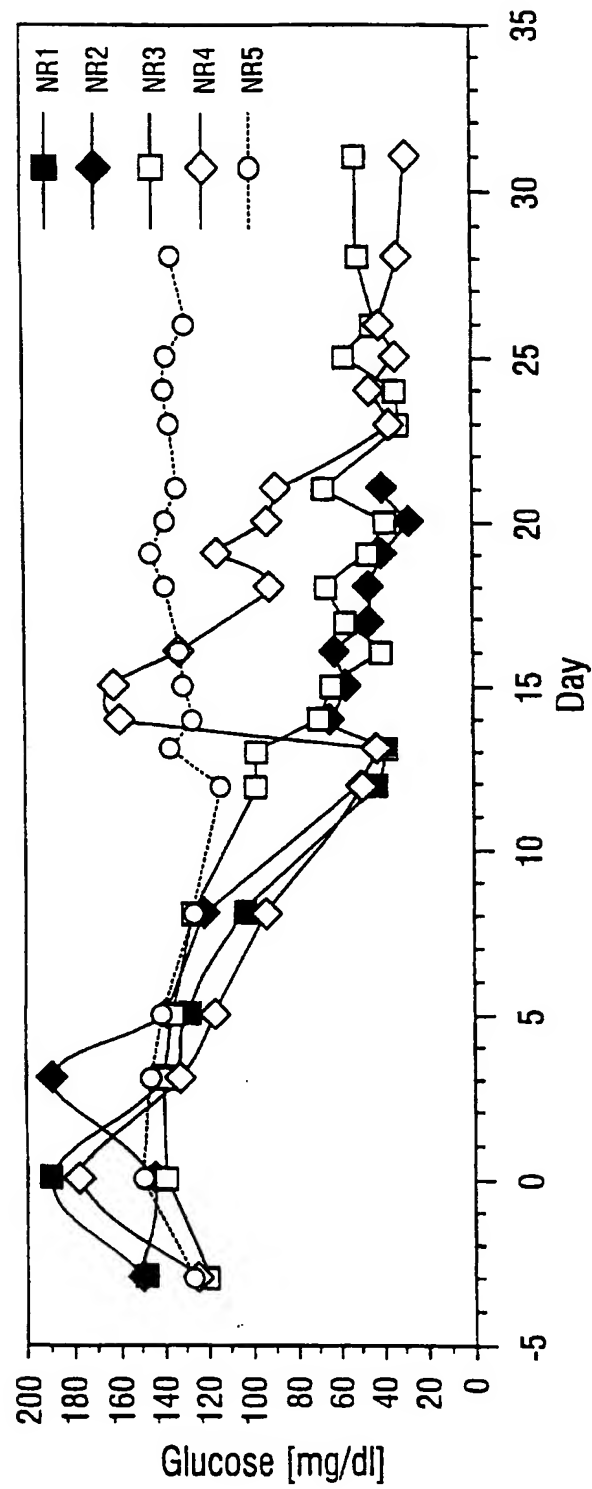


FIG. 6A

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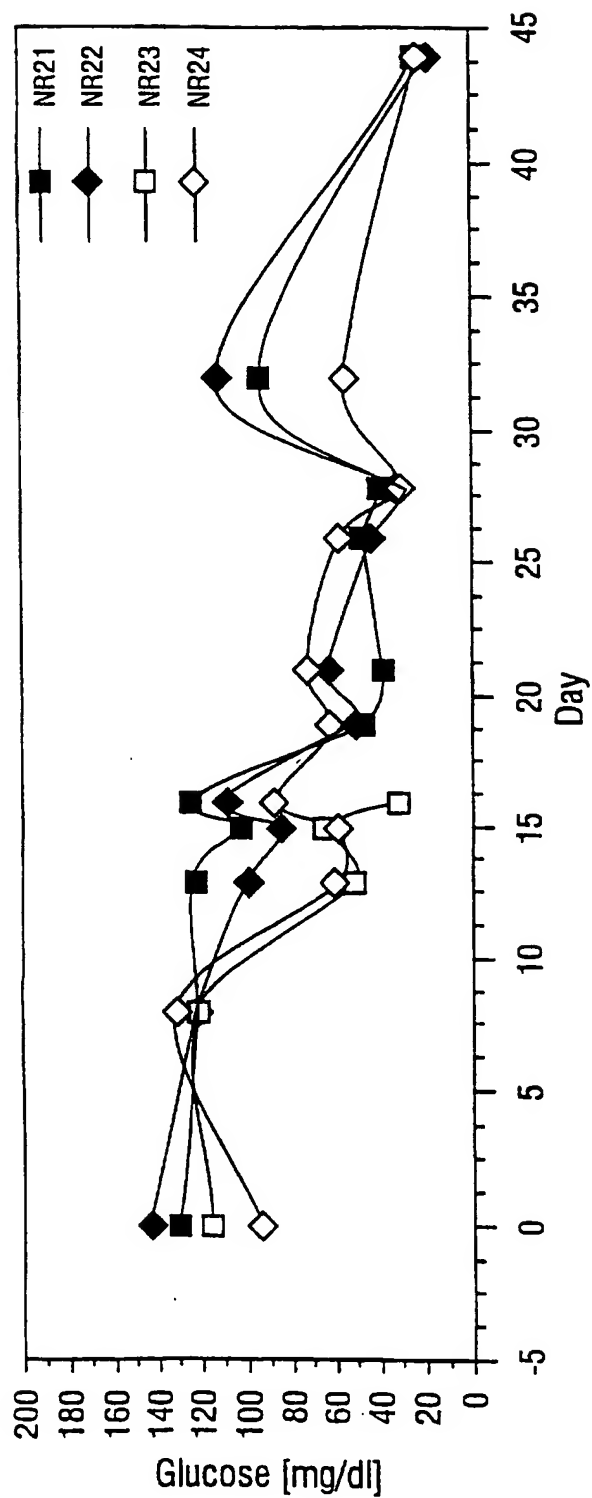


FIG. 6B

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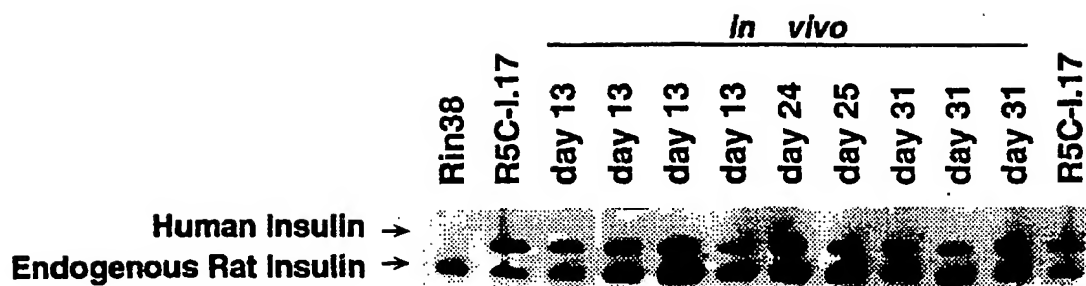


FIG. 7

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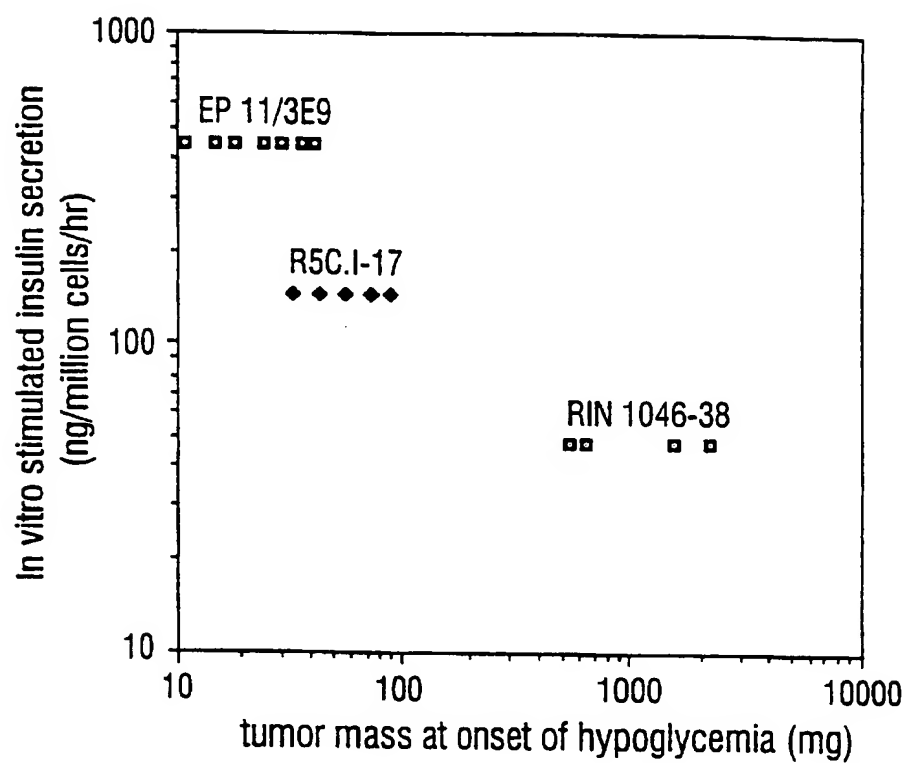


FIG. 8

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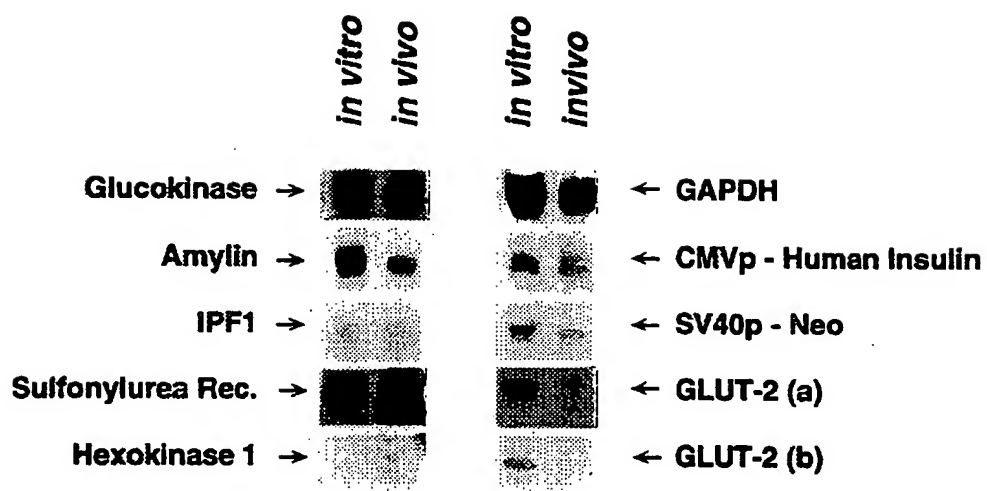


FIG. 9

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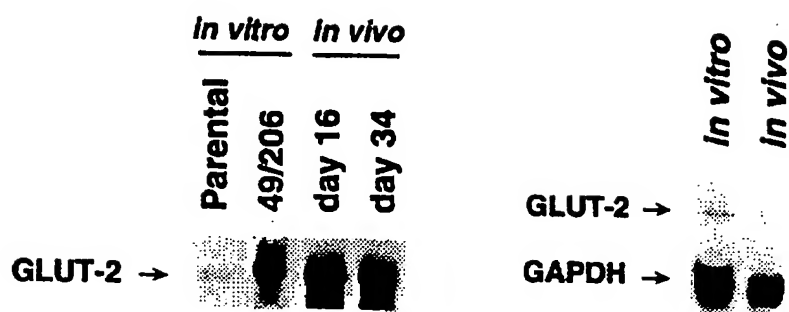


FIG. 10A

FIG. 10B

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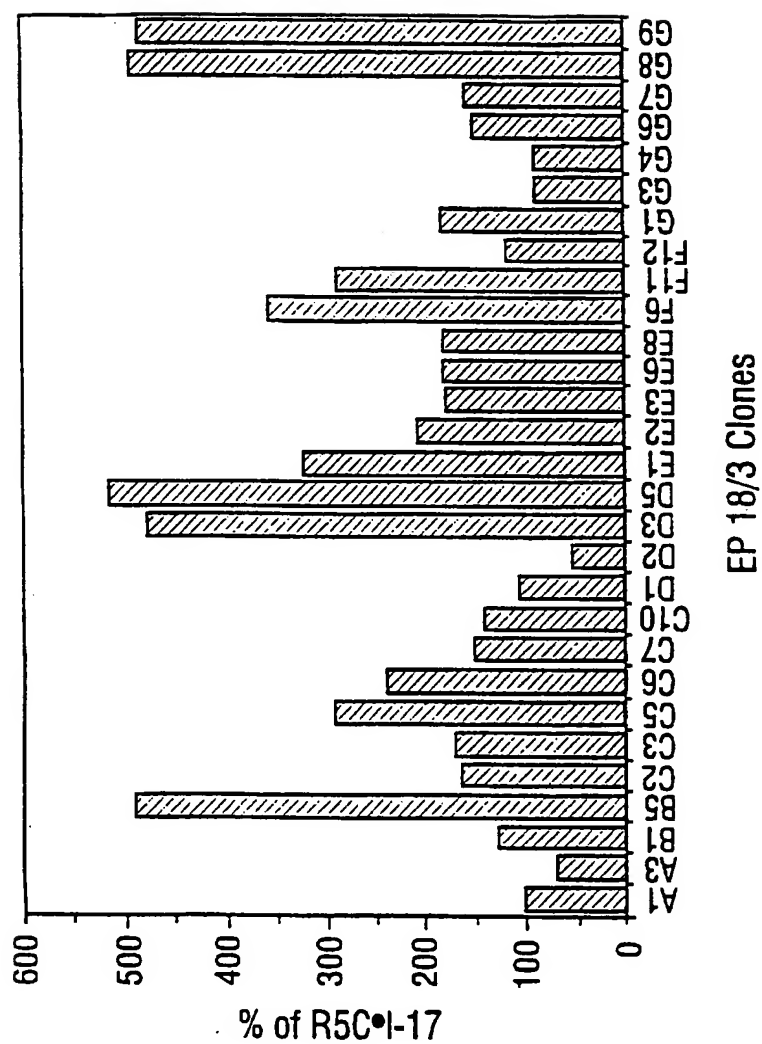


FIG. 11

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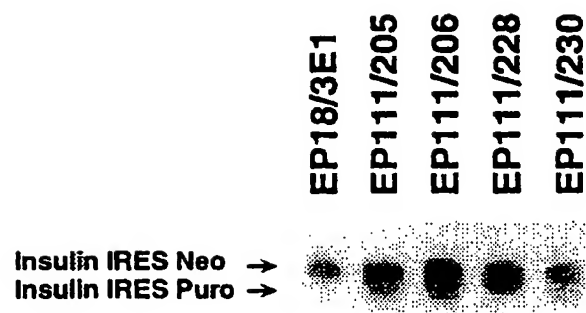


FIG. 12A

SUBSTITUTE SHEET (RULE 26)

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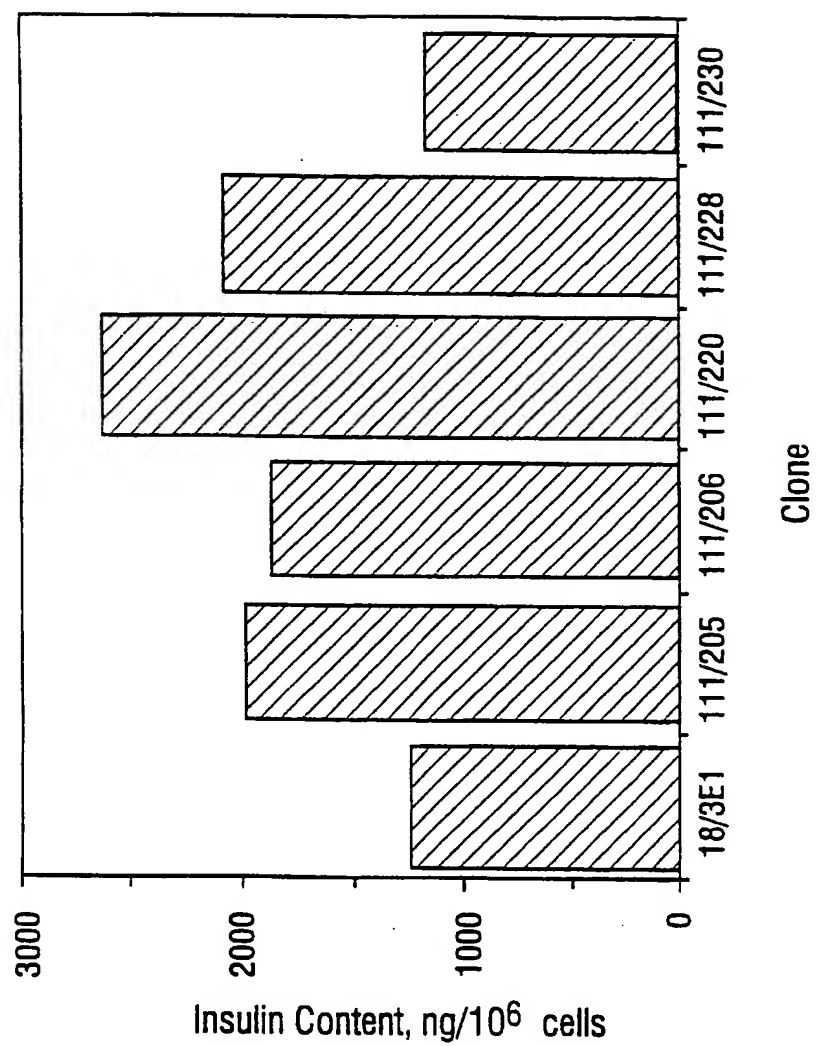


FIG. 12B

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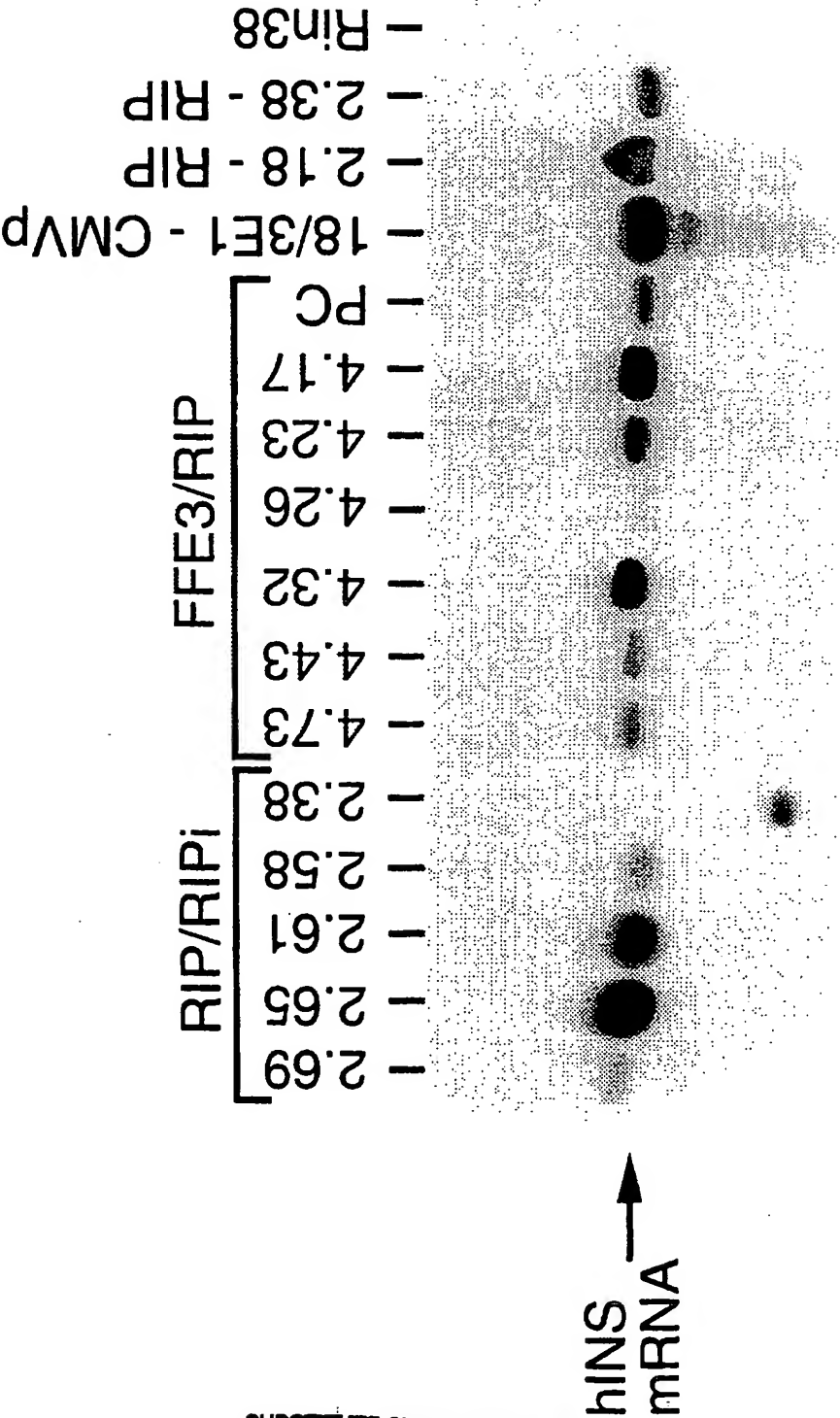


FIG. 13

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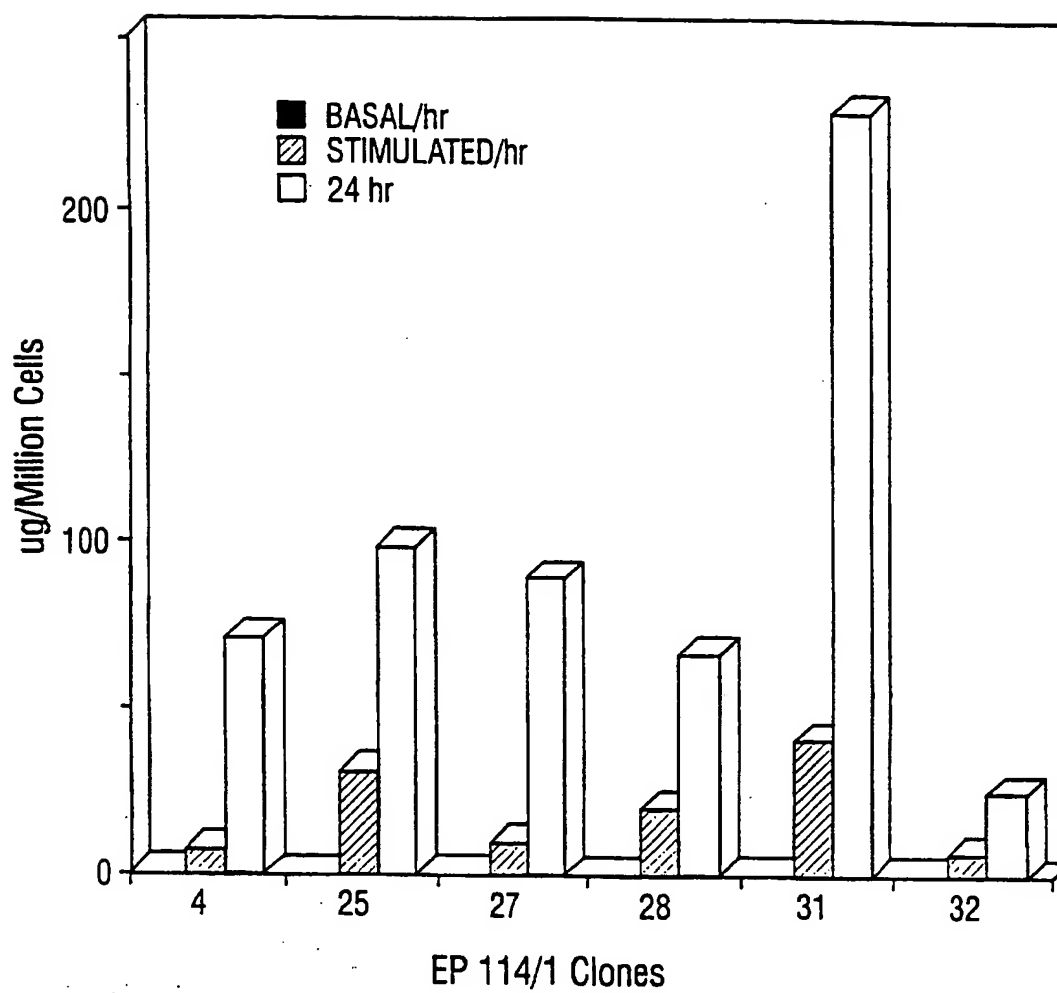


FIG. 14

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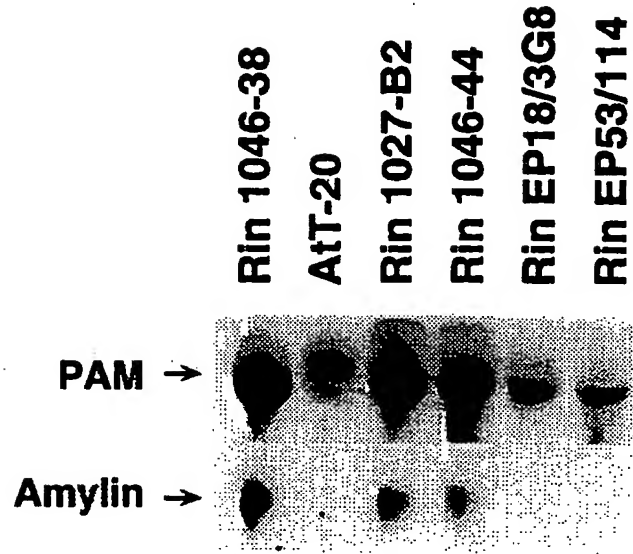


FIG. 15A

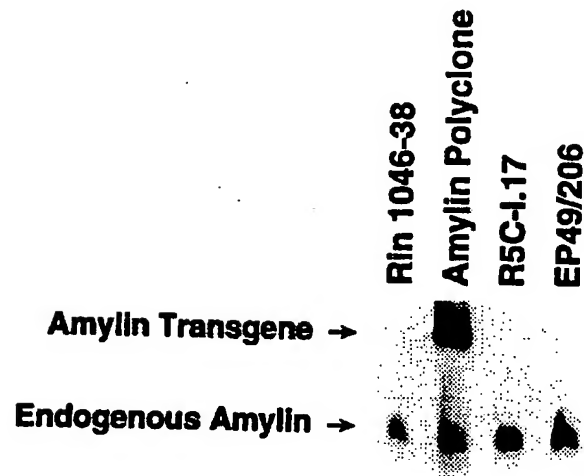


FIG. 15B

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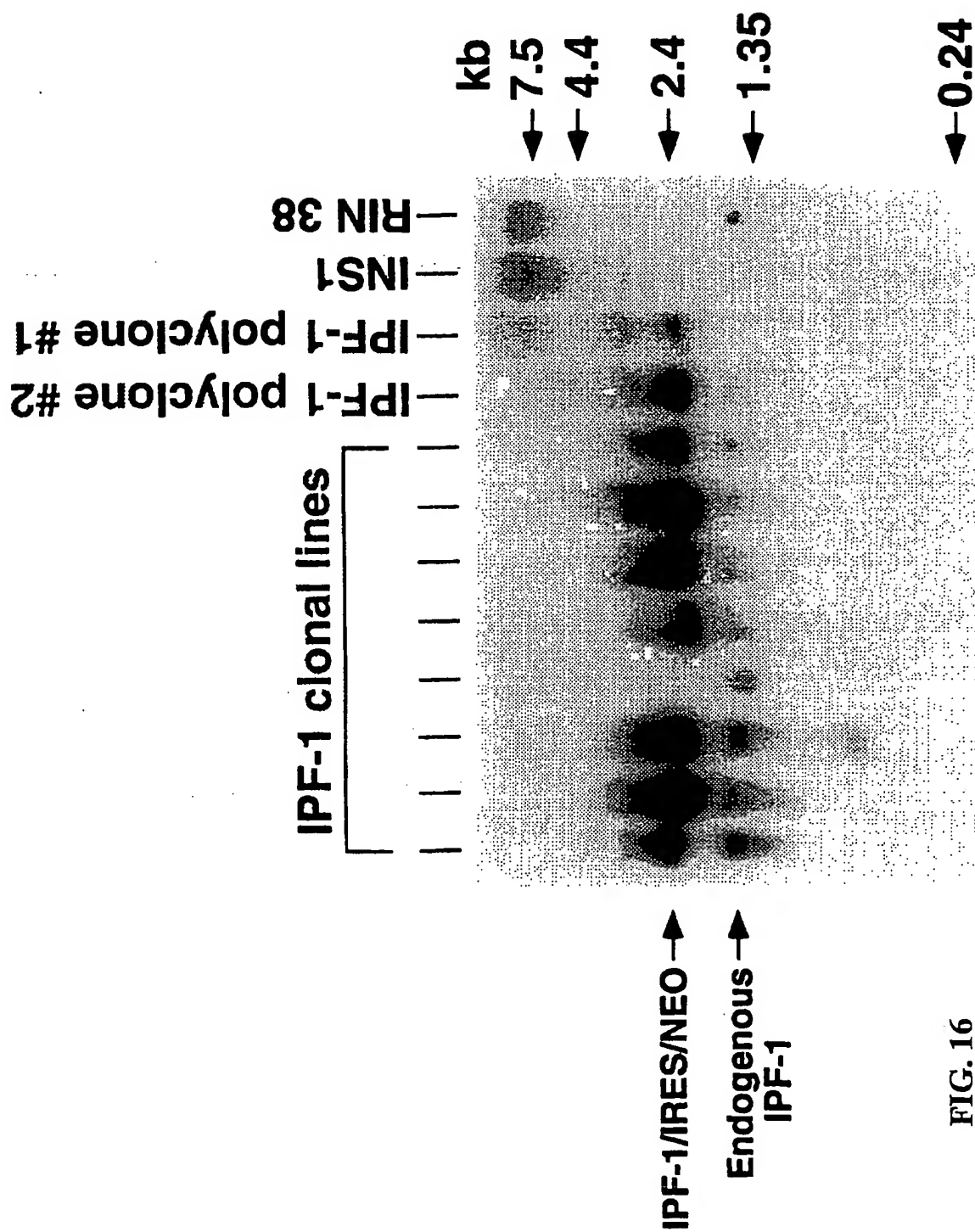


FIG. 16

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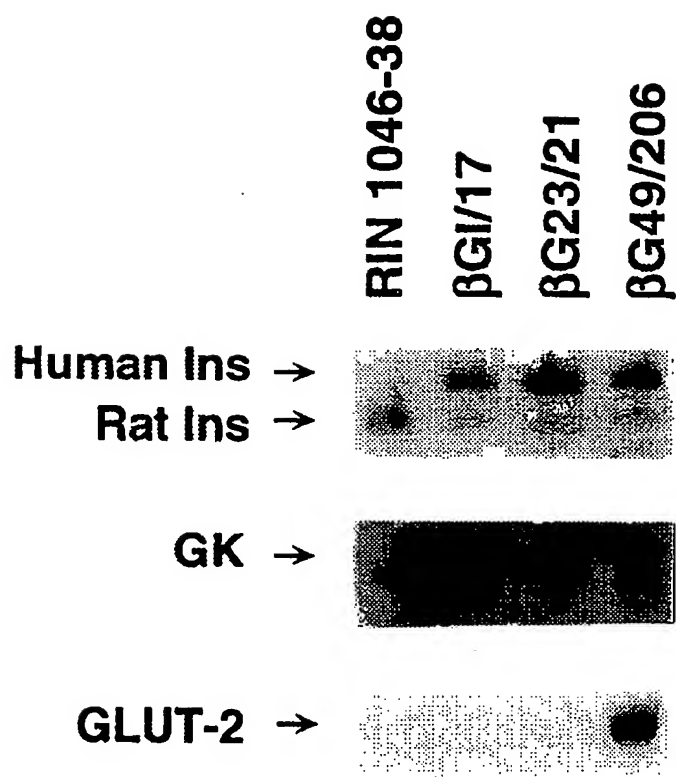


FIG. 17

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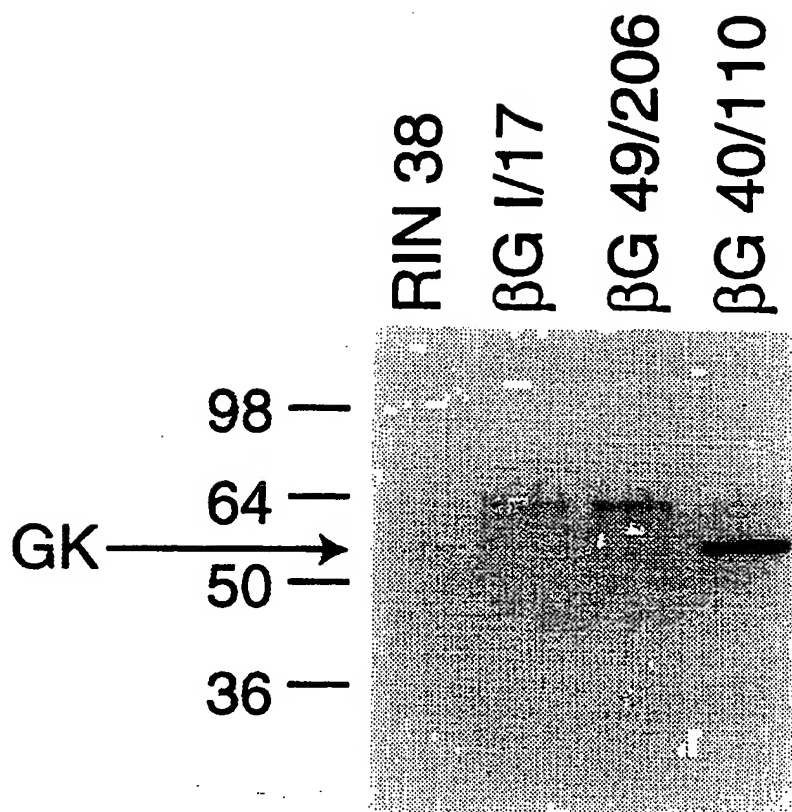


FIG. 18

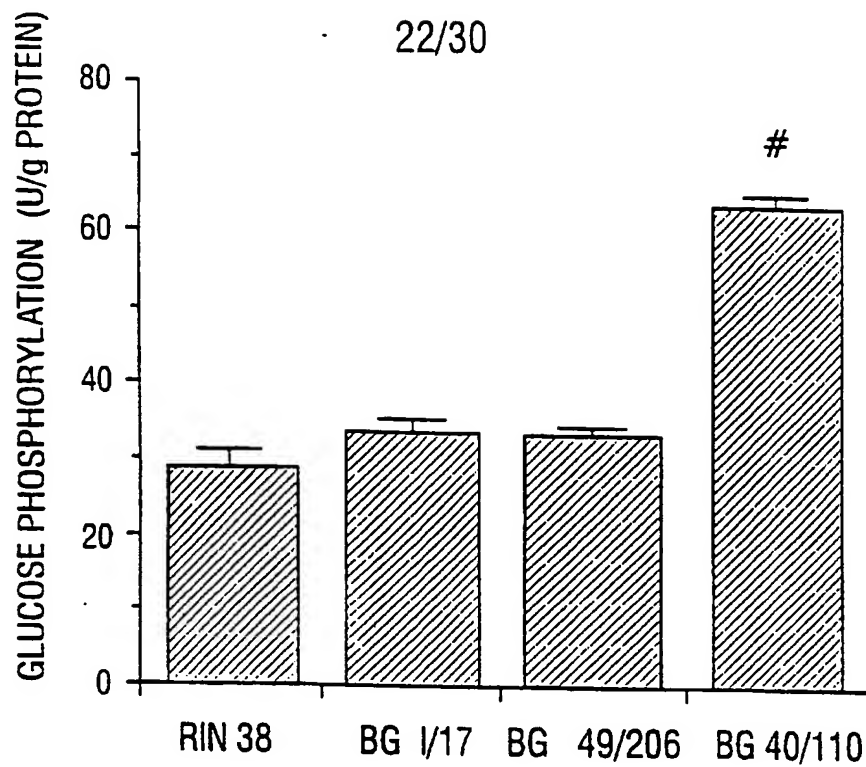


FIG. 19A

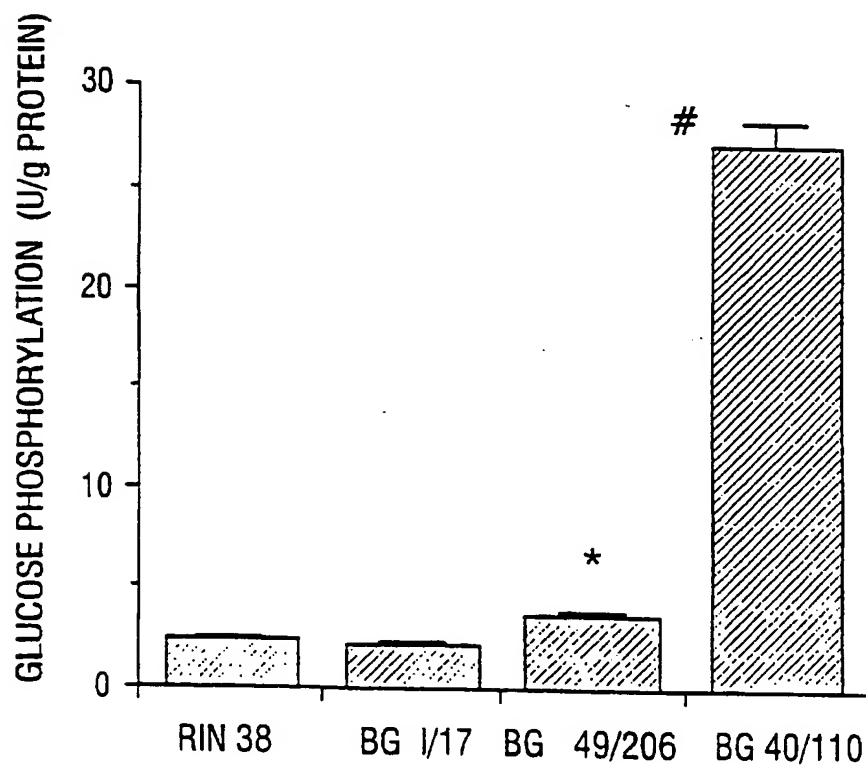


FIG. 19B

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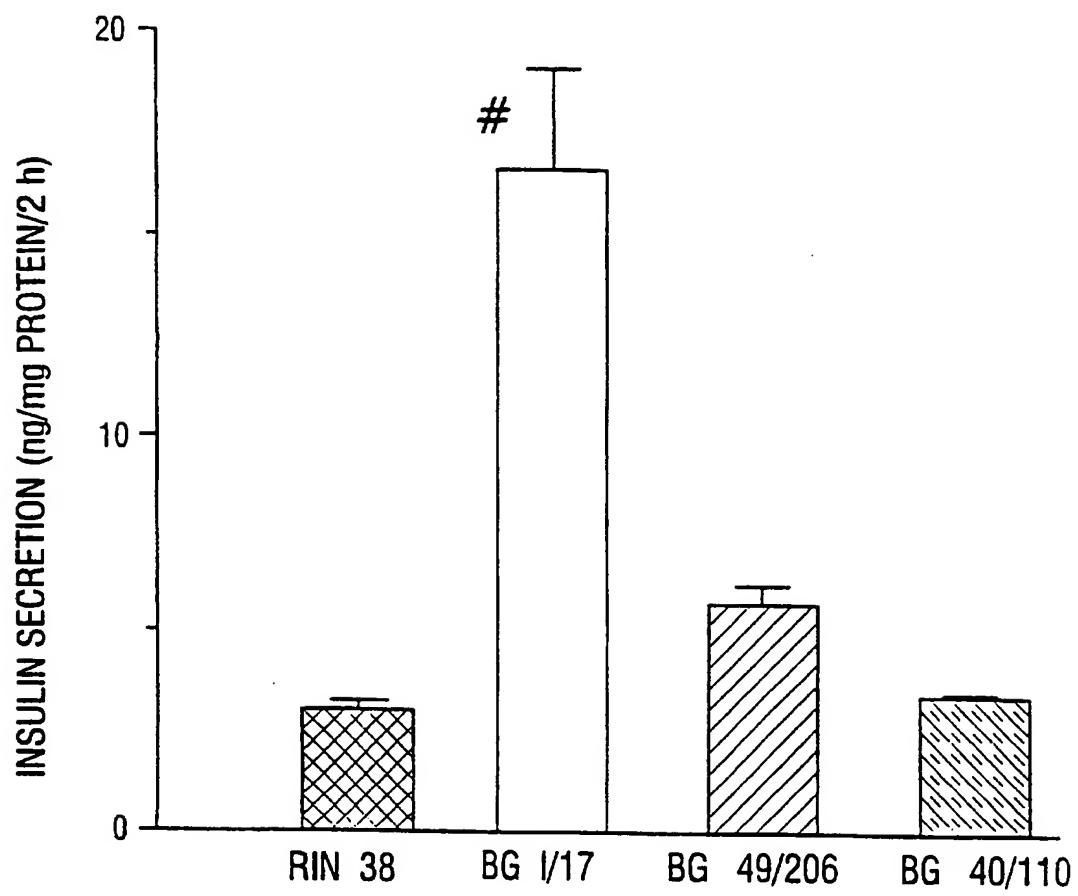


FIG. 20

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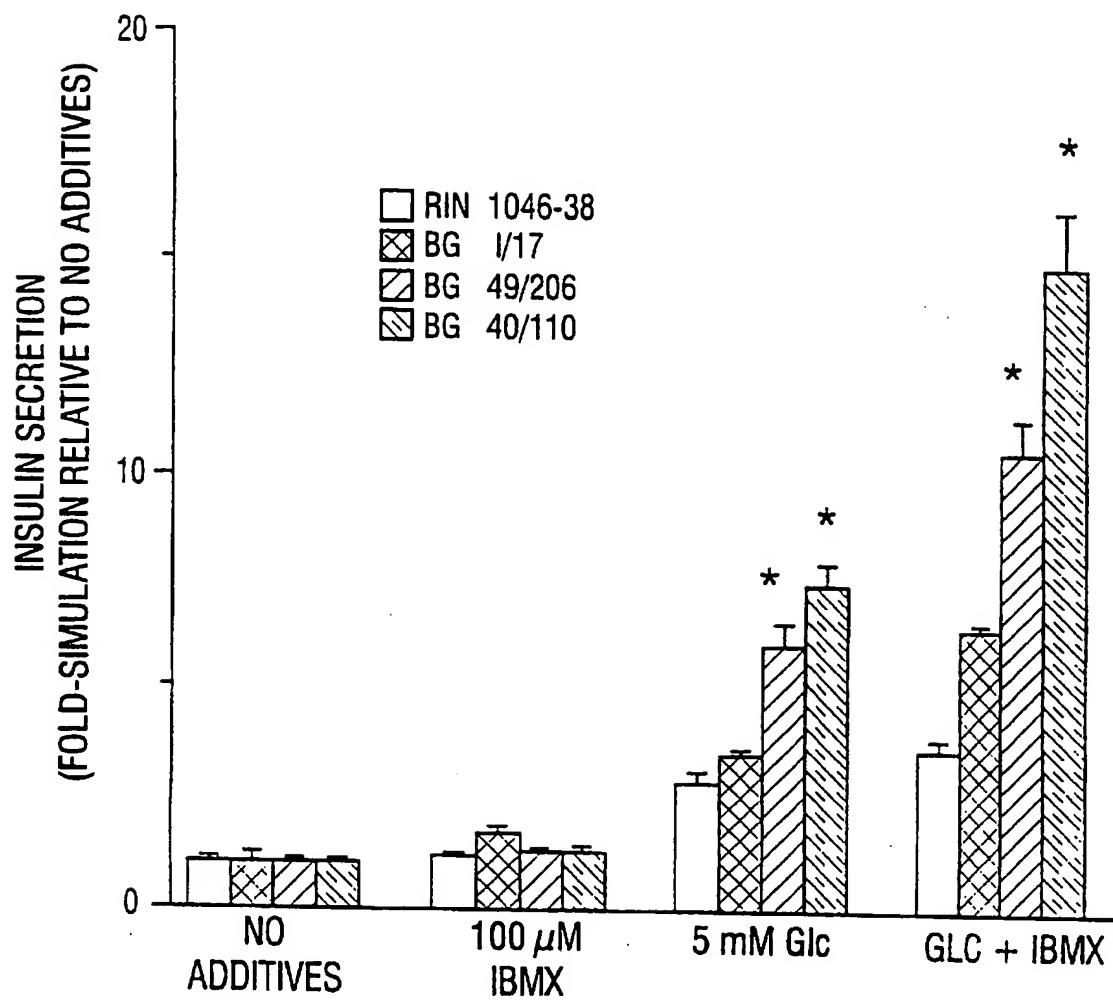


FIG. 21

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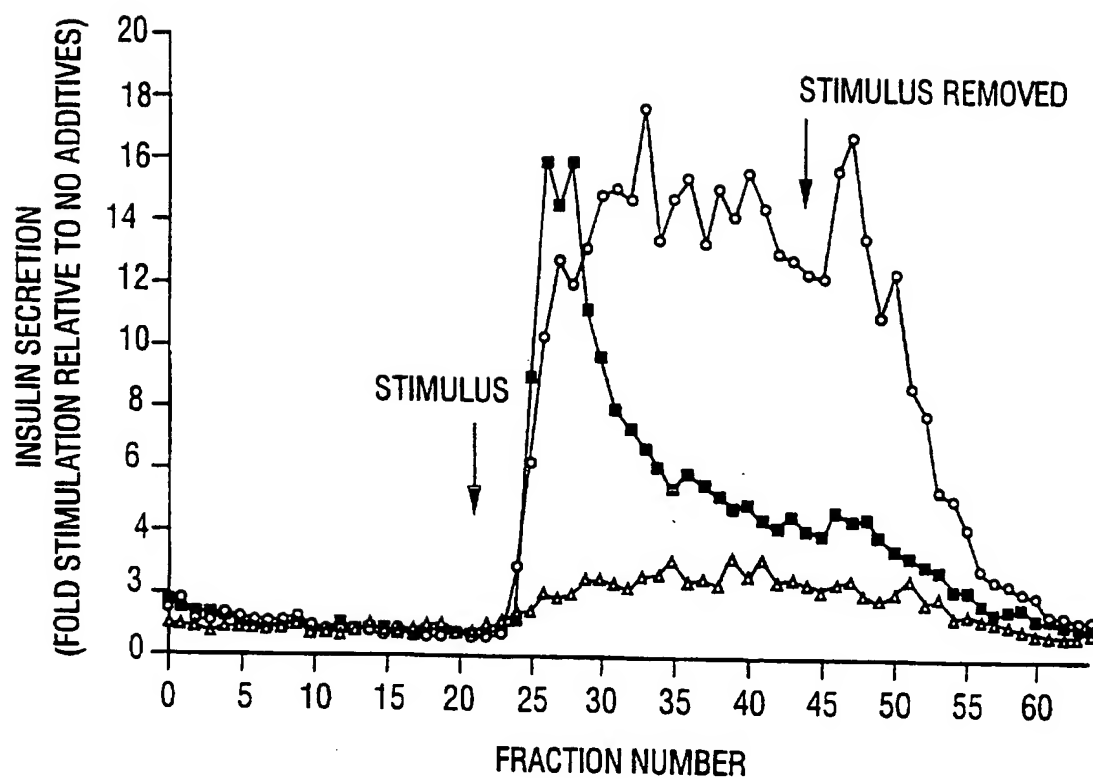


FIG. 22

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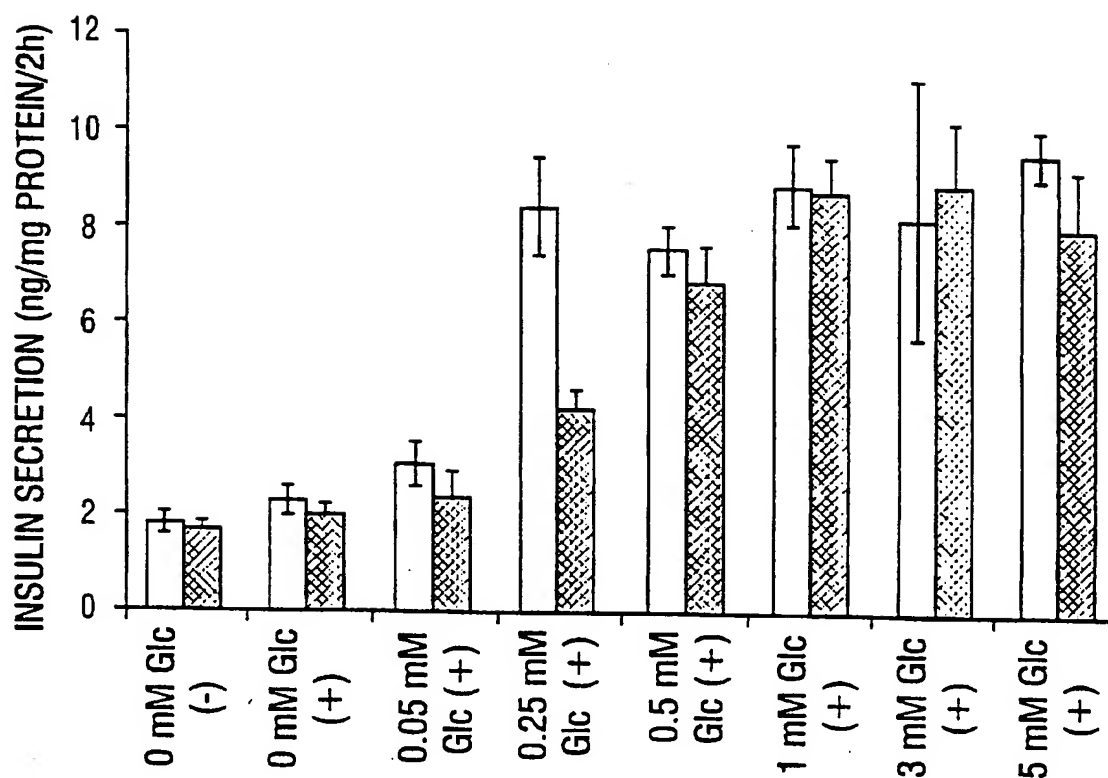


FIG. 23A

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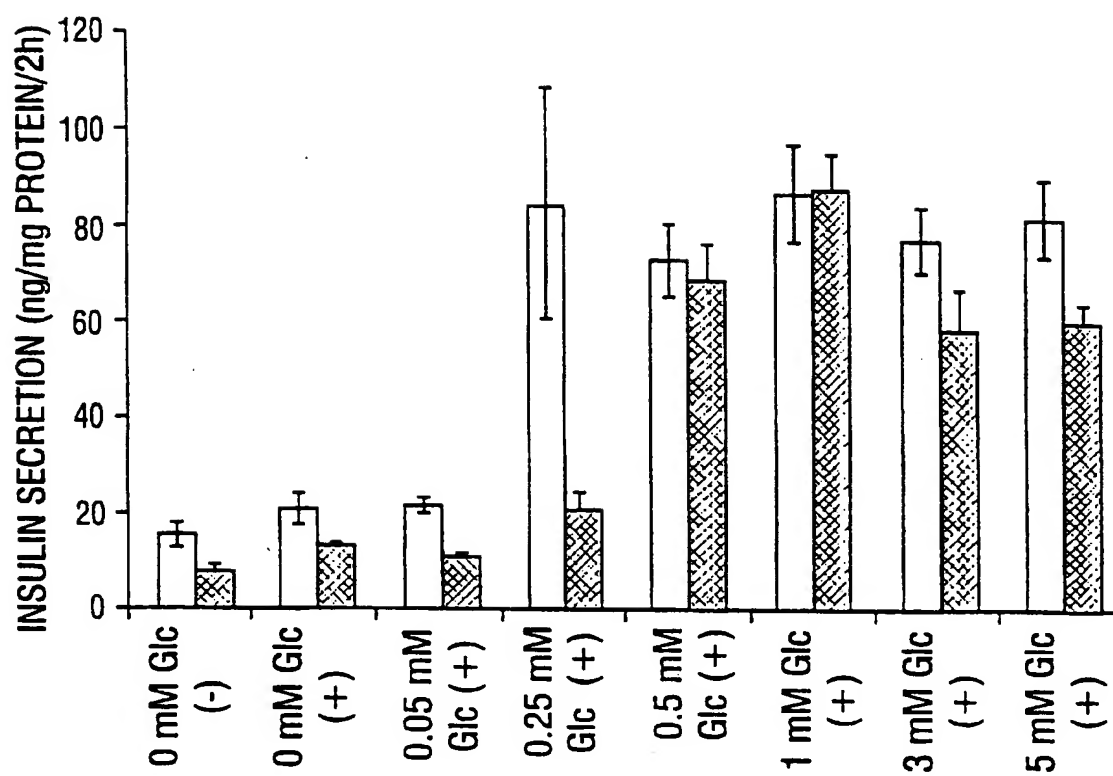


FIG. 23B

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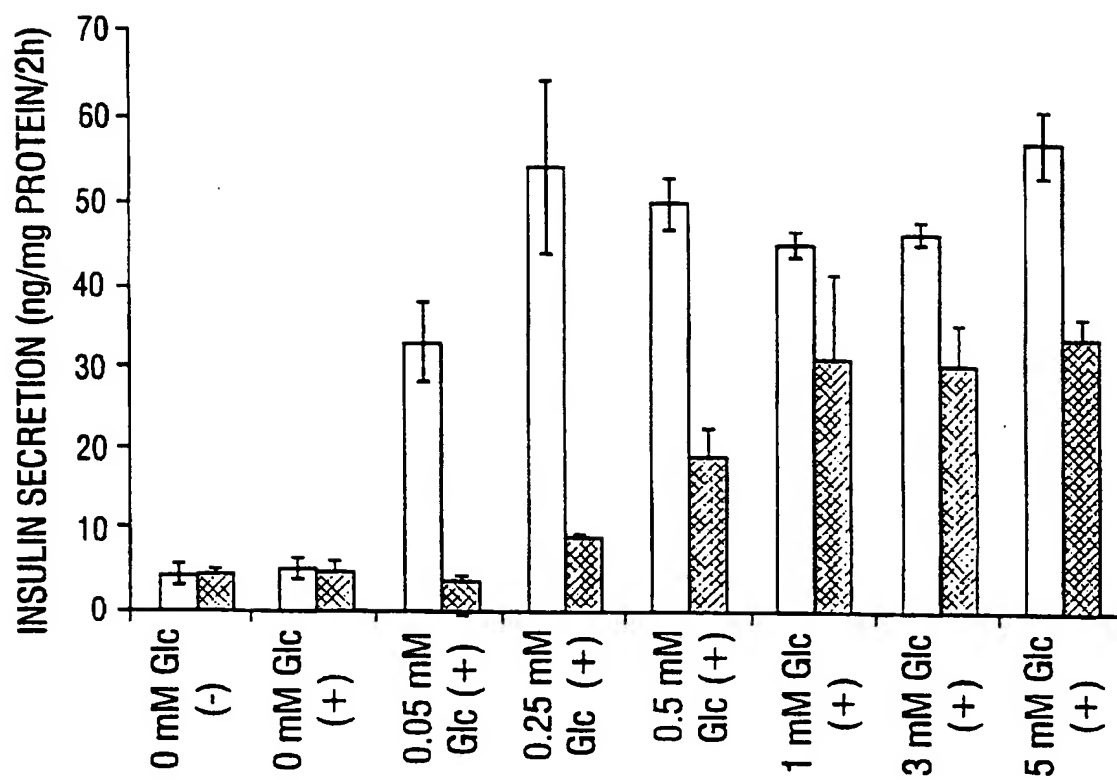


FIG. 23C

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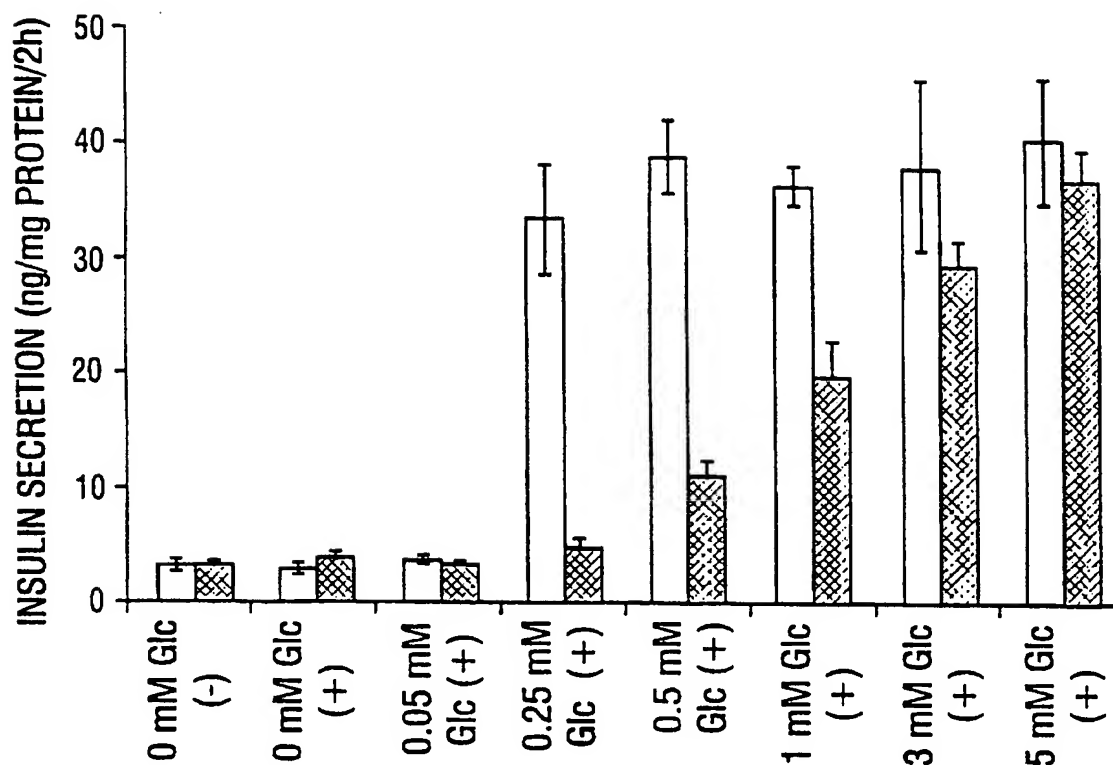


FIG. 23D

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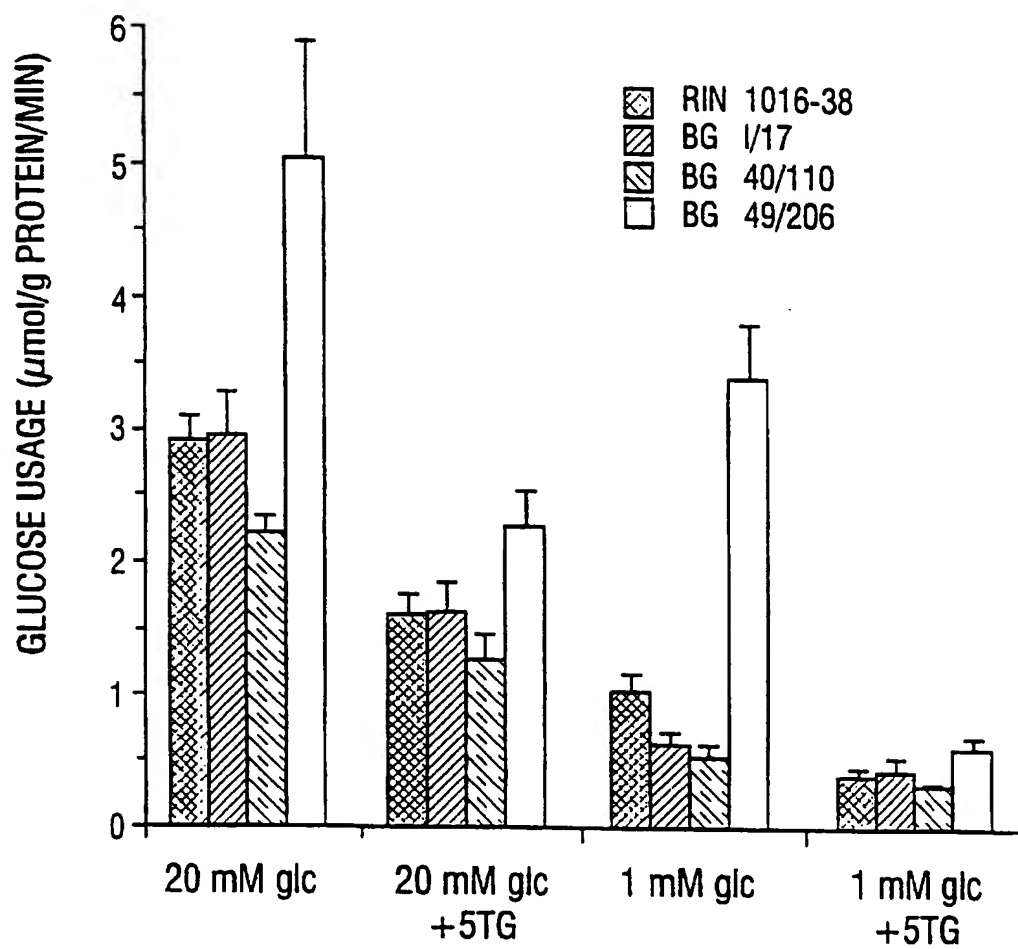


FIG. 24

INTERNATIONAL SEARCH REPORT

International Application No
PLI/US 97/00760

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6	C12N15/12	C12N15/16	C12N15/17	C12N15/18	C12N15/54
	C12N15/85	C12N5/10	C12N9/12	C07K14/47	C07K14/575
	C07K14/605	C07K14/61	C07K14/62	C12P21/02	A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 92 21756 A (UNIV TEXAS) 10 December 1992 see the whole document ---	1-72
A	WO 92 21979 A (UNIV TEXAS) 10 December 1992 see the whole document ---	1-72
A	WO 95 00644 A (UNIV TEXAS ;NEWGARD CHRISTOPHER B (US); GERARD ROBERT D (US)) 5 January 1995 see the whole document ---	1-72
-/--		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the international search

16 May 1997

Date of mailing of the international search report

27.05.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

International Application No
PL 1/US 97/00760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CELL, vol. 35, December 1983, CELL PRESS,CAMBRIDGE,MA,US;; pages 531-538, XP000673678 H.-P. H. MOORE ET AL.: "Expressing a human proinsulin cDNA in a mouse ACTH-secreting cell. Intracellular storage, proteolytic precessing, and secretion on stimulation" cited in the application see the whole document ---</p>	1-72
A	<p>PROC. NATL.ACAD SCI., vol. 80, January 1993, NATL. ACAD SCI.,WASHINGTON,DC,US;; pages 397-401, XP002031140 G.N. PAVLAKIS AND D.H. HAMER: "Regulation of a metallothionin-growth hormone hybrid gene in bovine papilloma virus" cited in the application see the whole document ---</p>	73-75
A	<p>PROC. NATL.ACAD SCI., vol. 91, November 1994, NATL. ACAD SCI.,WASHINGTON,DC,US;; pages 10967-10971, XP002031141 M.W. HEARTLEIN ET AL.: "Long-term production and delivery of human growth hormone in vivo" cited in the application see the whole document ---</p>	73-75
A	<p>DIABETES (1994), 43(3), 341-50 CODEN: DIAEAZ;ISSN: 0012-1797, March 1994, XP000673632 NEWGARD, CHRISTOPHER B.: "Cellular engineering and gene therapy strategies for insulin replacement in diabetes" see the whole document ---</p>	76-112
A	<p>TRANSPLANT. PROC. (1994), 26(2), 363-5 CODEN: TRPPA8;ISSN: 0041-1345, April 1994, XP002031142 FERBER, S. ET AL: "Molecular strategies for the treatment of diabetes" see the whole document ---</p>	76-112
A	<p>WO 94 20624 A (GENENTECH INC) 15 September 1994 see the whole document ---</p>	76-112
A	<p>WO 95 27512 A (BAYLOR COLLEGE MEDICINE) 19 October 1995 see the whole document ---</p>	76-112
-/--		

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/00760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 32740 A (UNISEARCH LTD ;TUCH BERNARD EDWARD (AU); SIMPSON ANN MARGARET (AU)) 7 December 1995 see the whole document -----	76-112

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/00760

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 76-112
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 76-112 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 97/00760

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9221756 A	10-12-92	US 5427940 A	27-06-95
		AT 145065 T	15-11-96
		AU 656213 B	27-01-95
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		EP 0687301 A	20-12-95
		JP 8508398 T	10-09-96

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/00760

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9527512 A	19-10-95	AU 2283495 A	30-10-95
		CA 2188675 A	19-10-95
		EP 0755268 A	29-01-97

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